Amino Acid Sensing in Hypothalamic Tanycytes via Umami Taste Receptors

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Table of Contents

Figures and Tables 4
Acknowledgements 5
Declaration 6
Summary 7
Abbreviations 8
1. Introduction 11
1.1. Regulation of energy homeostasis 12
1.1.1. Peripheral signals 12
1.1.2. Hypothalamic nuclei involved in energy homeostasis 16
1.1.3. Integration of metabolic state-related signals 23
1.1.4. Hypothalamic plasticity 25
1.1.5. Other neuronal mechanisms of food intake control 28
1.1.6. Glia and energy homeostasis 29
1.2. Hypothalamic tanycytes 32
1.2.1. Morphology and physiology 32
1.2.2. Functions 33
1.2.3. Potential role in food intake control 34
1.3. Amino acid sensing in the brain 35
1.3.1. Amino acid transport into the brain 36
1.3.2. Brain amino acid detection mechanisms 37
1.4. Amino acid receptors 38
1.4.1. Umami taste receptors 40
1.4.2. ATP signalling 43
1.4.3. P2 receptors 45
1.5. Taste receptors in tanycytes 45
1.6. Experimental aims 46
2. Hypothalamic tanycytes sense amino acids via a Ca^{2+} and ATP-dependent pathway 47
2.1. Introduction 47
2.2. Methods 48
2.2.1. Brain slices 48
2.2.2. Solutions and drug application 49
2.2.3. Imaging 50
2.2.4. ATP biosensing 52
2.2.5. Data analysis 52
2.2.6. Statistical analysis 53
2.3. Results 53
2.3.1. Tanycytes respond to L-amino acids with a Ca^{2+} wave 53
2.3.2. Tanycytes release ATP in response to L-amino acids 59
2.3.3. ATP is released via large conductance channels 63
2.4. Discussion 64
2.4.1. Tanycytes exhibit specific responses to different L-amino acids 65
2.4.2. Tanycyte ATP release could be used for tanycyte-to-neuron signalling 66

3. The identification of amino acid receptors in tanycytes 68
3.1. Introduction 68
3.2. Methods 69
3.2.1. Animals and brain slices 69
3.2.2. Solutions 70
3.2.3. Immunohistochemistry 70
3.2.4. Imaging 70
3.2.5. Data and statistical analysis 71
3.3. Results 71
3.3.1. Inosine 5'-monophosphate increases tanycyte sensitivity to L-arginine 71
3.3.2. Tas1r1 is expressed in mouse tanycytes 73
3.3.3. *Tas1r1*-null mice exhibit altered responses to L-amino acids 75
3.3.4. mGluR4, but not mGluR1, mediates tanycyte responses to L-alanine and partially to L-lysine 77
3.3.5. Elimination of Tas1r1/Tas1r3 and mGluR4 is not sufficient to block all tanycyte responses to L-amino acids 77
3.3.6. Mouse tanycytes express mGluR4 79
3.4. Discussion 80
3.4.1. Tanycytes use Tas1r1/Tas1r3 to detect L-arginine and L-lysine 80
3.4.2. Multiple receptors are required for tanycyte detection of L-amino acids 81
3.4.3. Physiological changes in amino acid levels in the third ventricle could potentially activate tanycytes *in vivo* 82
3.4.4. Male and female mice adapt differently to the loss of *Tas1r1* 83

4. Diet-related changes in feeding, metabolism and tanycyte sensitivity to amino acids 85
4.1. Introduction 85
4.2. Methods 87
4.2.1. Animals and tissue preparation 87
4.2.2. Diet 89
4.2.3. Experimental cages 89
4.2.4. Imaging 90
4.2.5. Data and statistical analysis 90
4.3. Results 92
4.3.1. Short-term fasting increases tanycyte sensitivity to L-alanine 92
4.3.2. Oral L-alanine supplementation does not alter food intake after short-term fasting 93
4.3.3. Amino acid-imbalanced diet changes tanycyte sensitivity to amino acids 95
4.3.4. Essential amino acid-imbalanced diet reduces preference for an L-alanine enriched drink in wild type mice 97
4.3.5. *Tas1r1*-null mice have reduced preference for L-alanine independently of essential amino acid intake 99
4.3.6. Amino acid-imbalanced diets only have minor effects on mouse metabolism in 24 hours 101
4.3.7. An alanine-rich diet affects mouse metabolism 104
4.4. Discussion 109
4.4.1. Tanycyte sensitivity to L-alanine is diet-dependent 110

4.4.2. Potential further tanycyte signalling adaptations to dietary changes 113
4.4.3. Changes in tanycyte signalling could be linked to metabolism.. 113
5. Discussion 116
5.1. Similarities between tanycytes and taste receptor cells 118
5.2. Tanycytes as amino acid sensors in the brain..... 120
5.3. A new role for L-alanine in rodent regulation of energy homeostasis121
5.4. Potential role for amino acid sensing in tanycyte neurogenesis..... 122
5.5. Future work 123
6. Conclusions..... 126
References 127
Appendix..... 150

Figures and Tables

Figure 1.1. Peripheral signals involved in the regulation of energy homeostasis 13
Figure 1.2. Hypothalamic circuits involved in the regulation of energy homeostasis 17
Figure 1.3. Hypothalamic integration of food-related signals 24
Figure 1.4. Amino acid-sensitive G protein-coupled receptor pathways 42
Figure 2.1. Set up for Ca^{2+} imaging and biosensing 51
Figure 2.2. Tanycytes respond to L-lysine 54
Figure 2.3. Tanycytes respond to L-arginine 55
Figure 2.4. Tanycytes respond to L-alanine 56
Figure 2.5. Tanycytes respond to L-serine 57
Figure 2.6. Tanycytes have low sensitivity to L-proline 59
Figure 2.8. Tanycyte responses to L-amino acids require ATP detection via P2 receptors 60
Figure 2.9. Tanycytes release ATP in response to L-amino acids 61
Figure 2.10. ATP travels down tanycyte processes 62
Figure 2.11. ATP is most likely released from tanycytes via Pannexin 1 after L-arginine application 63
Figure 2.12. Tanycyte ATP release in response to L-alanine requires CalHM1 64
Figure 3.1. IMP enhances tanycyte responses to L-arginine 72
Figure 3.2. Tanycytes express Tas1r1 74
Figure 3.3. Tas1r1/Tas1r3 is involved in tanycyte amino acid sensing. 76
Figure 3.4. Tanycytes use mGluR4 to detect some L-amino acids 78
Figure 3.5. mGluR4 is expressed in hypothalamic tanycytes 79
Figure 3.6. Proposed tanycyte amino acid signalling pathways 80
Figure 4.1. Fasting increases tanycyte sensitivity to L-alanine. 93
Figure 4.2. L-alanine supplementation in water does not affect food intake after feeding 95
Figure 4.3. Diet deficient in L-arginine and L-lysine increases tanycyte sensitivity to L-alanine 96
Figure 4.4. Diet deficient in L-arginine and L-lysine reduces preference for an L-alanine-enriched drink 98
Figure 4.5. Tas1r1-null mice have reduced preference for L-alanine 100
Figure 4.6. Diet composition affects metabolism 102
Figure 4.7. Food amino acid content does not affect feeding or metabolism in female mice over 24 hours 104
Figure 4.8. Increasing L-alanine content in the diet reduces light phase feeding in male and female mice 105
Figure 4.9. Dietary alanine has a minor effect on activity 107
Figure 4.10. An alanine-rich diet affects metabolism 108

Table 1. Hypothalamic nuclei and their functions. 22
Table 2. Diet-related changes in tanycyte responses to amino acids and metabolism. 111

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Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree, apart from part of the material in section 2.3.2 and data shown in Figure 3.1 E, which were previously submitted for a Master's degree by Research.

The work presented (including data generated and data analysis) was carried out by the author, except one of the experiments in sections 4.3.6 and 4.3.7, where Prof. Francis Ebling and Dr. Jo Lewis (University of Nottingham) performed parts of the experiment in the author's absence. The author was present at the start, middle and end of the experiment, remotely monitored the progress, and carried out the data analysis.

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Summary

Energy homeostasis is controlled in the hypothalamus. Hypothalamic tanycytes are a type of glial cell that lie in the wall of the third ventricle, between ventricular CSF and the key nuclei controlling feeding, and are potentially linked to this regulation. Tanycytes have been found to express sweet taste receptors; similar receptors exist for amino acids and could be present in tanycytes. Therefore, the aims of this work were to understand the tanycyte amino acid signalling process, determine the impact of diet on tanycyte function, and link tanycytes to energy homeostasis.

Using ratiometric Ca^{2+} imaging, I showed that tanycytes responded to amino acids; the responses also involved ATP release via pannexin 1 and CalHM1 from tanycyte cell bodies and down their processes towards the parenchyma. As IMP increased the amplitude of tanycyte responses to some amino acids, one of the receptors is Tas1r1/Tas1r3. The Tas1r1 subunit was expressed in mouse tanycytes, and *Tas1r1*-null mice showed sex-specific altered responses to arginine and lysine. mGluR4 antagonist MAP4 reduced the responses to alanine, and mGluR4 was shown to be present in mouse tanycytes. Tas1r1/Tas1r3 and mGluR4 are therefore two of the receptors responsible for tanycyte amino acid signalling.

Fasting and essential amino acid deprivation increased tanycyte sensitivity to alanine. Essential amino acid-deprived diet also reduced the intake of an alanine-enriched drink. While amino acid-imbalanced diets did not have strong effects on mouse metabolism over 24 hours, increasing dietary alanine content reduced feeding and increased metabolism in the light phase.

These results demonstrate for the first time that tanycytes can detect amino acids, and that they do so via two different pathways, similarly to taste receptor cells in the tongue. Tanycytes can send ATP signals to inform the hypothalamus about nutrient availability. The data also show that tanycyte amino acid sensitivity is diet-dependent. High alanine food has a satiating effect, which could be partially mediated by tanycytes, although a direct link cannot be confirmed at this stage.

Abbreviations

3V	Third ventricle
4-CPG	S-4-carboxyphenylglycine
α-MSH	α-melanocyte-stimulating hormone
A-	Alanine-deficient diet
A+	Alanine-enhanced diet
aCSF	Artificial cerebrospinal fluid
AgRP	Agouti-related peptide
AIDA	(*RS*)-1-aminoindan-1,5-dicarboxylic acid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPK	AMP-activated protein kinase
AP	Area postrema
APC	Anterior piriform cortex
ARC	Arcuate nucleus
BAT	Brown adipose tissue
BBB	Blood-brain barrier
BBG	Brilliant blue G
BDNF	Brain-derived neurotrophic factor
C2	Nutrient- and calorie-matched control diet
CalHM1	Calcium homeostasis modulator 1
cAMP	Cyclic adenosine monophosphate
CART	Cocaine- and amphetamine- related transcript
CaSR	Calcium-sensing receptor
CaTCh	Ca^{2+} permeable channelrhodopsin
CBX	Carbenoxolone
CCK	Cholecystokinin
CHOx	Carbohydrate oxidation
CLAMS	Comprehensive Laboratory Animal Monitoring System
CNTF	Ciliary neurotrophic factor
CPPG	(*RS*)-α-cyclo- propyl-4-phosphonophenylglycine
CRH	Corticotropin-releasing hormone
CSF	Cerebrospinal fluid
Cx43	Connexin 43
DIO	Diet-induced obesity
DMH	Dorsomedial hypothalamic nucleus
DRP1	Dynamin-related protein 1
DVC	Dorsal vagal complex
EE	Energy expenditure
ER	Oestrogen receptor
ERα	Oestrogen receptor-α
FGF	Fibroblast growth factor
Fox	Fat oxidation
GABA	γ- aminobutyric acid
GE	Glucose-excited neurons
GI	Glucose-inhibited neurons
GLP	Glucagon-like peptide
GLUT2	Glucose transporter 2
GnRH	Gonadotropin-releasing hormone
GPCR	G protein-coupled receptor

GPRC6A	G protein-coupled receptor class C group 6 member A
Hcrt	Hypocretin
HEPES	4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid
HFD	High-fat diet
HP	High-protein
ICV	Intracerebroventricular
iGluR	Ionotropic glutamate receptor
IMB	Amino acid-imbalanced diet
IMP	Inosine 5'-monophosphate
IP_3	Inositol-1,4,5-trisphosphate
KA	Kainate
K_{ATP}	ATP-sensitive K^+ channel
LepR	Leptin receptor
LH	Lateral hypothalamic nucleus
LPD	Low-protein diet
MAP4	S-2-amino-2-methyl-4-phosphonobutanoic acid
MBH	Mediobasal hypothalamus
MC3R	Melanocortin receptor 3
MC4R	Melanocortin receptor 4
MCH	Melanin-concentrating hormone
ME	Median eminence
mGluR	Metabotropic glutamate receptor
mTOR	Mammalian target of rapamycin
NAc	Nucleus accumbens
NFκB	Nuclear factor kappa B
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NPY	Neuropeptide Y
NTS	Nucleus of the solitary tract
Orx	Orexin
OXM	Oxyntomodulin
OXTR	Oxytocin receptor
Panx1	Pannexin 1
PBS	Phosphate-buffered solution
PFA	Paraformaldehyde
PIP2	Phosphatidylinositol-4,5-bisphosphate
PLC-β2	Phospholipase C β2
POMC	Proopiomelanocortin
PP	Pancreatic polypeptide
PVN	Paraventricular nucleus
PYY	Peptide tyrosine tyrosine
RER	Respiratory exchange ratio
RK-	Arginine- and lysine-deficient diet
ROI	Region of interest
RuR	Ruthenium Red
SCN	Suprachiasmatic nucleus
SON	Supraoptic nucleus
T3	Triiodothyronine
T4	Thyroxine
TRPM5	Transient receptor potential cation channel subfamily M member 5

TTX	Tetrodotoxin
VCO_2	Carbon dioxide production
VGlut2	Vesicular glutamate transporter 2
VMH	Ventromedial hypothalamic nucleus
VO_2	Oxygen consumption
VTA	Ventral tegmental area

1. Introduction

The impact of food choices on health and wellbeing has been known for millennia: as famously said by Hippocrates, “Let food be thy medicine and medicine be thy food”. Unfortunately, most people in the Western cultures seem to not follow this rule; the World Health Organization has recognized obesity as a disease since 1948 (James and Rigby 2010), and it has gradually developed into an epidemic (Mitchell et al. 2011). 70 years later, scientists and medical professionals have not yet found a full explanation of what underlying processes in the body and the brain control feeding, the storage of energy and its expenditure through metabolism. These processes are collectively called energy homeostasis and are now known to be primarily controlled in the hypothalamus, with influences from the periphery as well as other sites of the brain (Sainsbury et al. 2002).

While it seems obvious from the modern neuroscience point of view, the involvement of the brain in the regulation of energy homeostasis was not apparent until the early 20th century. Previously, it was believed that the stomach was in full control of hunger, although alternative theories about a “hunger centre in the blood” had been proposed (Mayer 1953). However, in 1901 Fröhlich observed excessive weight gain in a patient who suffered from a tumour in the pituitary region, linking the central nervous system with metabolic functions for the first time (Bruch 1939). This and several other cases prompted further studies in the field, and in 1940 Hetherington and Ranson discovered that lesions in the medial hypothalamus consistently caused obesity in male and female rats (Hetherington and Ranson 1940). Similar research continued, including Anand and Brobeck revealing in 1951 that bilateral destruction of the lateral hypothalamic nucleus induced anorexia (Anand and Brobeck 1951). A decade later, Kennedy concluded that the hypothalamus regulated food intake and activity in response to changes in body fat, as well as caloric intake and blood glucose level (Kennedy 1961). Current knowledge extends his observations, connecting hormonal signals from the periphery and blood nutrient levels with specific cell populations in the hypothalamus and the brainstem, which then lead to adjustments in feeding and energy expenditure.

Hypothalamic tanycytes, a type of non-neuronal cell lining the third ventricle, are potentially linked to the regulation of energy homeostasis. Tanycytes have recently been found to respond to changes in glucose concentration in the ventricular cerebrospinal fluid, suggesting that they may monitor the nutritive status of the brain (Frayling et al. 2011). Moreover, our laboratory has shown that tanycytes sense glucose through the same sweet taste receptors (Tas1r2/Tas1r3) that are used in the taste buds of the tongue to detect sugar and sweeteners (Benford et al. 2017). Receptors for another taste modality – umami (the savoury flavour of L-glutamate for humans and most non-aromatic L-amino acids for rodents) – are closely related to the sweet taste receptors (Zhao et al. 2003), proposing the idea that the umami taste receptor may be involved in tanycyte amino acid sensing. This dissertation explores the potential contribution of tanycytes as hypothalamic amino acid sensors in the control of feeding and energy expenditure.

1.1. Regulation of energy homeostasis

While the discoveries relating brain injury to changes in food consumption were unexpected at the time, it is now widely accepted that the hypothalamus integrates metabolic information from the digestive tract and sensory organs, as well as locally produced signals. The combined effects of central and peripheral signals are then used to determine feeding-associated behaviours and adjust long-term energy expenditure. The presently established aspects of the control of energy homeostasis are described here in detail.

1.1.1. Peripheral signals

Initially, the information about the metabolic status of an organism comes from its digestive system and adipose tissue (Figure 1.1).

When the stomach empties, the epithelial cells in the stomach lining produce ghrelin, a hormone that acts on neurons to stimulate hunger (Asakawa et al. 2001). The expression of ghrelin in the stomach is increased by fasting but reduced in obese mice by anorexigenic hormones leptin and interleukin-1β (Asakawa et al. 2001). Ghrelin is transported into the brain over the median eminence (ME), a circumventricular organ within the hypothalamus containing

fenestrated capillaries, which gives the area around the third ventricle improved access to circulating hormones (Schaeffer et al. 2013). Ghrelin increases neuronal production of neuropeptide Y (NPY), which in turn increases food

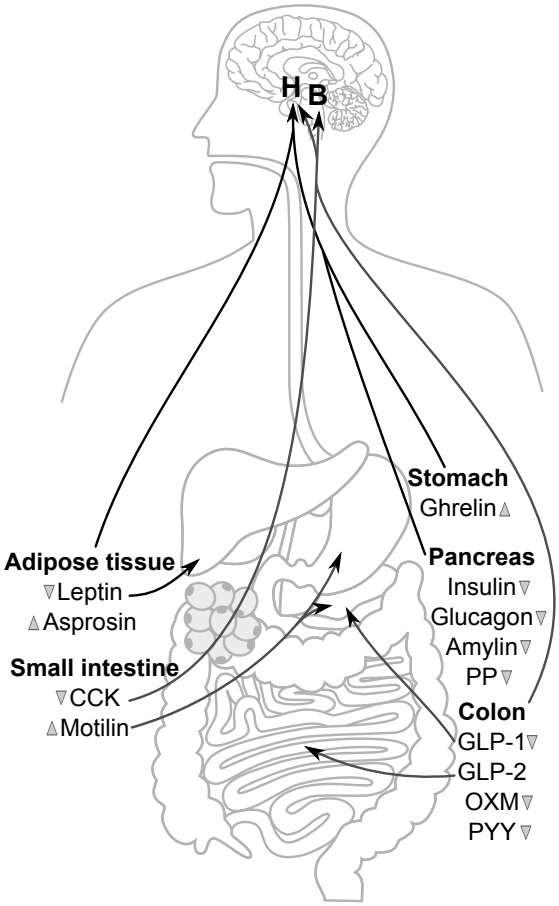

Figure 1.1. Peripheral signals involved in the regulation of energy homeostasis. *Adipose tissue cells produce leptin and asprosin, both detected in the hypothalamus. Stomach secretes orexigenic ghrelin, while the pancreas produces a range of anorexigenic hormones and peptides, all of which also affect neuronal activity in the hypothalamus. Peptides from the small and large intestine project to the hypothalamus and brainstem, as well as other parts of the digestive system. Grey triangles next to peptide names indicate their effect on food intake: triangle pointing 'up' represents an increase in feeding, 'down' represents a decrease. B, brainstem; H, hypothalamus; CCK, cholecystokinin; PP, pancreatic polypeptide; GLP-1, glucagon-like peptide-1; GLP-2, glucagon-like peptide-2; OXM, oxyntomodulin; PYY, peptide tyrosine tyrosine.*

intake and decreases energy expenditure (Asakawa et al. 2001).

Leptin is produced by the adipose tissue and acts to reduce food intake. Leptin regulates energy homeostasis by inactivating orexigenic NPY/agouti-related peptide (AgRP) neurons while activating anorexigenic proopiomelanocortin (POMC)/cocaine- and amphetamine- related transcript (CART) neurons in the hypothalamus (Schwartz et al. 1996, Cheung et al. 1997). This is achieved by

the binding of leptin to leptin receptors (LepR). Leptin also regulates the brain reward system by inhibiting dopaminergic neurons in the ventral tegmental area (VTA) to reduce the motivation for feeding (Hommel et al. 2006).

Unfortunately, a constantly high level of circulating leptin, as seen in many obese individuals, can lead to a dramatic reduction in leptin detection, which is referred to as leptin resistance and is a common cause for metabolic syndrome (Frederich et al. 1995). Two potential mechanisms for central leptin resistance are a decrease in leptin transport into its sites of action within the brain by tanycytes of the ME and the suppression of leptin signalling by an increase in circulating inhibitory molecules. Impaired leptin transport through the ME has been observed in obese mice, where leptin accumulated in the ME without entering the third ventricle (Balland et al. 2014), and the ratio of cerebrospinal fluid (CSF)/serum leptin is decreased in obesity (Caro et al. 1996).

The peripheral effects of leptin are the promotion of energy expenditure by increasing sympathetic nerve signalling to brown adipose tissue (BAT) and the modulation of gluconeogenesis in the liver by increasing parasympathetic nerve activity (Bell et al. 2017). Leptin has also been shown to suppress responses to sweet stimuli in the tongue (Kawai et al. 2000).

Another hormone produced by the adipose tissue has been recently discovered and termed asprosin (Romere et al. 2016). Asprosin secretion is induced by fasting and leads to hepatic glucose release and increased appetite. The latter effect is mediated by inhibition of hypothalamic POMC neurons, and neutralisation of asprosin in the blood with specific antibodies reduces food intake and body weight (Duerrschmid et al. 2017).

The pancreas produces insulin in response to increased blood glucose. Insulin has an anorexigenic effect as hypothalamic neurons expressing insulin receptors co-express POMC, which is a precursor for melanocortins, known to decrease appetite while increasing energy use (Benoit et al. 2002). The administration of insulin into the hypothalamus leads to a dose-dependent decrease in food intake and sustained weight loss in a range of species (Woods et al. 1979, Schwartz et al. 1992, Chavez et al. 1995). Moreover, the loss of

insulin receptors in the brain results in increased body weight and susceptibility to diet-induced obesity (Brüning et al. 2000).

The pancreas also produces glucagon, amylin, and pancreatic polypeptide (PP), all of which are anorexigenic (Suzuki et al. 2010). Glucagon secretion is induced by hypoglycaemia, and peripheral administration of glucagon reduces meal size and total food intake in rats (Geary and Smith 1982). Amylin is coreleased with insulin and has been demonstrated to reduce meal size and duration (Lutz et al. 1995). PP reduces food intake by acting on the Y4 receptor in the brainstem and hypothalamus, as well as via the vagus nerve (Asakawa et al. 2003). Circulating PP concentration changes with food ingestion (Wisén et al. 1992).

A number of peptides related to the regulation of food intake are released from the small and large intestines.

Motilin, a 22-amino acid peptide secreted by the small intestine, regulates interdigestive motility, gallbladder contractions, and the secretion of enzymes from the stomach and pancreas (Itoh 1997). It has also been reported to stimulate growth hormone secretion. Motilin is closely related to ghrelin, and their receptors exhibit 50% identity with each other (Asakawa et al. 2001).

Cholecystokinin (CCK) is produced in the small intestine after food ingestion, reaching a peak concentration in the blood plasma within 15 minutes and reducing further food intake (Liddle et al. 1985). The two CCK receptor subtypes, CCK1 and CCK2, can be found in several areas of the brain, including the brainstem and hypothalamus (Honda et al. 1993), although the anorectic effect is mostly mediated via vagal afferents (Zarbin et al. 1981). Continuous CCK administration does not affect food intake (Crawley and Beinfeld 1983). Interestingly, CCK is also expressed in the taste buds and has been shown to modulate bitter taste sensitivity and transduction in mice (Yoshida et al. 2017).

Glucagon-like peptide-1 (GLP-1) is synthesized and released in the distal part of the small intestine and the colon in response to nutrients to suppress short-term food intake in rats (Rüttimann et al. 2009). It is also produced in the

nucleus of the solitary tract (NTS) of the brainstem, and acts as a neurotransmitter to depolarise neurons in the hypothalamus (Merchenthaler et al. 1999). GLP-1 is involved in glycaemic control as it stimulates insulin secretion and has a stimulatory effect on pancreatic β cells (Egan et al. 2003). Conversely, glucagon-like peptide-2 (GLP-2) has no effects on food intake, but chronic administration has been shown to have an intestinal trophic effect (Drucker et al. 1996). Another peptide in this family, oxyntomodulin (OXM), reduces food intake and increases energy expenditure in humans and rodents, although it has low affinity for the GLP-1 receptor (Cohen et al. 2003).

Peptide tyrosine tyrosine (PYY) is a 36-amino acid lower gut hormone which binds to the hypothalamic Y2 receptor, leading to a reduction in food intake (Batterham et al. 2002). PYY concentration in blood plasma is low during fasting but increases 1–2 hours after food ingestion and remains elevated for several hours, reflecting the size and nutrient proportions of the meal (Adrian et al. 1985). A study in mice has shown that PYY also increases energy expenditure independently of food intake (Boey et al. 2008).

A common feature of the peripheral peptides described above is that they all, either directly (via blood plasma) or indirectly (via vagus nerve or by stimulating the release of other peptides), send information about changes in the nutritive state to the brain, particularly to the brainstem and the hypothalamus (Figure 1.1).

1.1.2. Hypothalamic nuclei involved in energy homeostasis

The hypothalamus is the area of the brain that receives and integrates signals from the whole body, including the rest of the brain, about the homeostatic state of the organism. It is subdivided into three regions: periventricular, medial and lateral, different in their morphology and functions. The periventricular and medial hypothalamic zones contain nuclei involved in the regulation of homeostatic behaviours such as hunger, thirst, thermoregulation, circadian rhythms, and reproduction (Simerly 2015). A summary of hypothalamic nuclei and their functions is presented in Table 1, and the connections between the nuclei are shown in Figure 1.2.

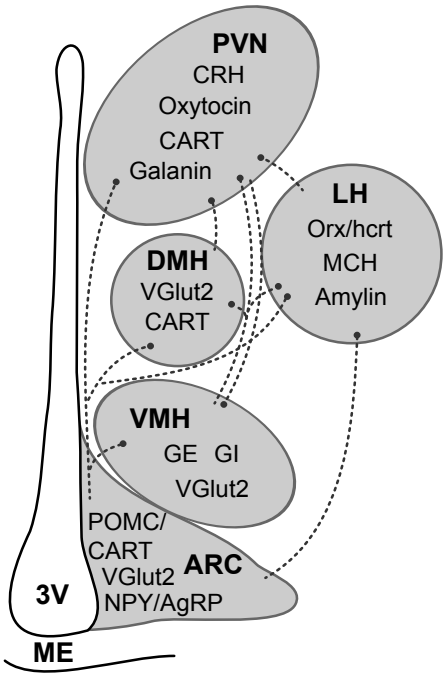

Figure 1.2. Hypothalamic circuits involved in the regulation of energy homeostasis. *The arcuate nucleus contains POMC/CART, NPY/AgRP and VGlut2 neurons that are directly involved in the regulation of energy balance. These neuronal populations project into other hypothalamic nuclei. The ventromedial hypothalamus has glucose-excited and glucose-inhibited neurons, as well as VGlut2 neurons. The dorsomedial hypothalamus has strong connections with other nuclei and integrates their signals. The lateral hypothalamus contains orexin/hypocretin and MCH neurons, both promoting positive energy balance, as well as anorexigenic amylin-producing neurons. The paraventricular nucleus neurons produce CRH and oxytocin to reduce food intake and galanin, which increases preference for high-fat foods. Dashed lines show projections between nuclei. 3V, third ventricle; AgRP, agouti-related peptide; ARC, arcuate nucleus; CART, cocaine- and amphetamine-regulated transcript; CRH, corticotropin-releasing hormone; DMH, dorsomedial hypothalamic nucleus; GE, glucose-excited neurons; GI, glucose-inhibited neurons; LH, lateral hypothalamus; MCH, melanocortin concentrating hormone; ME, median eminence; NPY, neuropeptide Y; orx/hcrt, orexin/hypocretin neurons; POMC, proopiomelanocortin; PVN, paraventricular nucleus; VGlut2, vesicular glutamate transporter 2; VMH, ventromedial hypothalamic nucleus.*

The ME is a circumventricular organ located in the basal hypothalamus containing fenestrated capillaries and tanycytes, which are heavily involved in the transport of blood-born substances such as gut peptides, hormones and nutrients into the arcuate nucleus (ARC) (Langlet et al. 2013, Balland et al. 2014). Thus, the ARC is considered to be outside the blood-brain barrier (BBB) (Ciofi 2011). The ARC contains the main neuronal populations responsible for regulating energy homeostasis: anorexigenic POMC/CART-producing neurons, and orexigenic NPY/AgRP-producing neurons (Hahn et al. 1998). The populations are localized in adjacent subsets and interact with each other in an asymmetric manner (Roseberry et al. 2004).

ARC POMC/CART neurons produce the precursor to an agonist to the melanocortin receptors 3 and 4 (MC3R, MC4R), the α-melanocyte-stimulating hormone (α-MSH), and the deletion of the *Pomc* gene results in hyperphagia and obesity (Yaswen et al. 1999). Intracerebroventricular (ICV) administration of a synthetic α-MSH analogue Melanotan II increases body temperature and oxygen consumption, as well as expression of uncoupling proteins in brown adipose tissue and muscle (Murphy et al. 2000, Cettour-Rose and Rohner-Jeanrenaud 2002, Hamilton and Doods 2002). Inactivation of MC4R has the opposite effects (Marie et al. 2000, Small et al. 2001, Baran et al. 2002). MC3R knockout mice are obese but their food intake is reduced, suggesting that MC4R modulates the hypophagic action of α-MSH (Butler et al. 2000).

Central administration of CART peptide reduces feeding in fasted rodents within 1 hour after the injection, as well as inhibits NPY-induced food intake in satiated rats (Kristensen et al. 1998). CART also acts on the dopaminergic system and is therefore associated with food- and drug-related rewards (Vicentic and Jones 2007).

While the expression of POMC and CART mostly overlaps in the ARC, the distribution of CART mRNA is much wider than that of POMC throughout the hypothalamus: CART is also found in the paraventricular nucleus (PVN), periventricular nucleus, dorsomedial nucleus (DMH), and lateral hypothalamus (LH) (Li et al. 2002). This distribution pattern has been observed in both mice and humans (Elias et al. 2001).

ARC POMC/CART neurons are considered to be slow in their response to feeding. Another anorexigenic population of cells expressing the vesicular glutamate transporter 2 (VGlut2) has been identified in ARC and demonstrated to rapidly suppress feeding (Fenselau et al. 2017). A subpopulation of these neurons (around 40%) also express POMC and CART, while others produce kisspeptin or no neuropeptides at all (Fenselau et al. 2017). ARC VGlut2 neurons are activated by oxytocin but inhibited by direct inputs from AgRP neurons and fasting (Fenselau et al. 2017). VGlut2 is also expressed in various neuronal subpopulations throughout the hypothalamus, but precise functions of

glutamatergic signalling originating from each hypothalamic nucleus are yet to be defined (Ziegler et al. 2002).

NPY/AgRP neurons respond to caloric deficiency, and their firing is negatively correlated with food ingestion, constantly increasing between meals but suddenly dropping as food becomes available (Mandelblat-Cerf et al. 2015). The orexigenic peptide NPY is widely expressed throughout the brain, with the main population of NPY/AgRP neurons involved in energy homeostasis located in the ARC (O'Donohue et al. 1985). ICV administration of NPY promotes feeding and fat storage while reducing signalling to BAT (Billington et al. 1991). Acute activation of ARC NPY/AgRP neurons also causes insulin resistance via peripheral actions on muscle-related genes in BAT (Steculorum et al. 2016). Chronic administration of NPY rapidly induces obesity (Raposinho et al. 2001).

AgRP is more sensitive to changes in metabolic state than POMC, so it may act to ensure a rapid response to the reduced availability of food in the melanocortin system (Harrold et al. 1999). AgRP neurons inhibit oxytocin secreting neurons to stimulate food intake (Atasoy et al. 2012). They also project via the amygdala into the bed nucleus of stria terminalis to suppress territorial aggression and reduce fear (Padilla et al. 2016). Additionally, AgRP directly inhibits neurons producing the reproductive hormone kisspeptin during metabolic deficiency, acting as one of the links between nutrition and fertility (Padilla et al. 2017). Thus, AgRP neurons are considered to be a major coordinator of behavioural responses to the metabolic state (Padilla et al. 2016).

The ventromedial hypothalamic nucleus (VMH) has been long considered to be the satiety centre of the brain: stimulation of neurons in the VMH inhibits feeding, while lesions in this area are associated with hyperphagia and weight gain (Stellar 1954). The VMH expresses leptin receptors and has direct connections with the PVN, LH, and DMH (Elmquist et al. 1998, Elmquist et al. 1999). Some neurons in the VMH, as well as ARC and LH, are known to be either glucose-excited (GE) or glucose-inhibited (GI) (Wang et al. 2004). The GE neurons increase, and the GI neurons decrease their action potential frequency as the level of extracellular glucose increases.

VMH GE neurons are inhibited by leptin and modulated by insulin depending on the concentration of extracellular glucose, while ARC GE neurons exhibit no leptin sensitivity and a variable response to insulin (Spanswick et al. 1997, Wang et al. 2004). ARC GE neurons, located in the lateral ARC, are activated via an ATP-sensitive K^+ channel (K_{ATP})-dependent pathway (Wang et al. 2004). NPY hyperpolarizes GE neurons and prevents them from firing; α-MSH increases their action potential frequency, and functional evidence suggests that ARC GE neurons are, in fact, a subpopulation of the POMC neurons (Ibrahim et al. 2003, Wang et al. 2004).

ARC GI neurons are located in the medial part of the ARC and most of them are NPY neurons (Fioramonti et al. 2007). While the exact pathway of ARC GI activation has not yet been clarified, a model has been proposed for VMH GI neurons: a decrease in glucose concentration induces VMH GI neuron activity by an increase in the AMP:ATP ratio within the cells, which activates AMP-activated protein kinase (AMPK) to increase nitric oxide (NO) production; moreover, the binding of NO to its receptor, soluble guanylyl cyclase, leads to cyclic GMP production, further increasing AMPK activation to a level that reduces Cl^- channel conductance, resulting in the depolarisation of the neuron (Murphy et al. 2009). In addition, activation of oestrogen receptors (ER) blunts the responses to low glucose in VMH GI neurons by inhibiting AMPK (Santiago et al. 2016). The AMPK/Cl^- channel-dependent activation pathway does not apply to LH GI neurons, which have been suggested to use tandem-pore (also known as two-pore) K^+ channels instead (Burdakov et al. 2006), although another group have shown that mice lacking this channel still exhibited glucose inhibition of LH neurons (Guyon et al. 2009).

The DMH is extensively interconnected with other hypothalamic nuclei and the brainstem (Horst and Luiten 1986, Thompson et al. 1996). Lesions in this region result in hypophagia and weight loss (Dalton et al. 1981). The DMH contains insulin and leptin receptors (Corp et al. 1986, Elmquist et al. 1998). Neurons in the DMH also participate in the regulation of stress-related cardiovascular parameters: heart rate, blood pressure and respiratory rate (Stotz-Potter et al. 1996).

The LH was originally known as the feeding centre of the brain, as stimulation of this nucleus increases food intake, while its destruction reduces feeding and causes weight loss (Anand and Brobeck 1951, Delgado and Anand 1952). Orexin/Hypocretin (orx/hcrt) neurons of the LH are associated with energy homeostasis (Sakurai et al. 1998, Thannickal et al. 2000). Orx neurons produce conserved neuropeptides called orexins: orxA and orxB (also termed hcrt1 and hcrt2) (De Lecea et al. 1998). Central administration of orx stimulates food consumption, and their production is reduced with feeding (Sakurai et al. 1998). Orx receptors are similar to the Y2 receptors (Sakurai et al. 1998).

Orx/hcrt neuron activity correlates with wakefulness and is reduced during sleep (Estabrooke et al. 2001). Humans and animals with low numbers of orx/hcrt neurons exhibit abnormal sleeping patterns such as narcolepsy (Thannickal et al. 2000). These neurons have also been described to modulate thermogenesis, cardiovascular and gastrointestinal functions (Berthoud et al. 2005). Interestingly, while hunger and hypoglycaemia activate orx/hcrt neurons to induce foraging, food consumption rapidly decreases orx/hcrt activity; orx/hcrt neurons are therefore associated with locomotion and food seeking behaviour rather than feeding (González et al. 2016).

Another population of LH neurons is known as the melanin-concentrating hormone (MCH)-producing neurons. MCH neurons are activated by glucose, and MCH induces sleep and suppresses metabolism, which implies that these neurons promote energy conservation after a period of high energy intake (Burdakov et al. 2005). Mice lacking MCH are hypophagic and exhibit a lean phenotype (Shimada et al. 1998).

Conversely, while orx/hcrt and MCH neurons are associated with increased feeding and energy preservation, some neurons in the LH express the pancreatic peptide amylin, which reduces food intake and positively modulates the effects of leptin in the hypothalamus (Li et al. 2015).

The periventricular nucleus contains neurons expressing NPY (Pelletier et al. 1984), somatostatin (Kiss et al. 1988), and kisspeptin, which regulates female reproduction by acting on gonadotropin-releasing hormone (GnRH)-secreting

neurons (Adachi et al. 2007). While not directly involved in energy homeostasis, the periventricular nucleus has close connections with ARC and ME (Kiss et al. 1988).

Table 1. *Hypothalamic nuclei and their functions.*

Region	Nucleus	Functions
Periventricular	Periventricular	Growth and sex hormone regulation
	Suprachiasmatic	Circadian rhythm generation
	Paraventricular	Integration of signals related to energy homeostasis
	Arcuate	Production of POMC, CART, NPY and AgRP
Medial	Medial preoptic	Thermoregulation (Tan et al. 2016)
	Anterior hypothalamic	Initiation of sexual behaviour (Mathews and Edwards 1977)
	Dorsomedial	Regulation of insulin production; cardiovascular stress responses
	Ventromedial	Inhibition of feeding; glucosensing
	Premammillary	Control of reproduction (Malpaux et al. 1998)
	Mammillary bodies	Locomotion, memory (Vann and Aggleton 2004)
	Posterior hypothalamic	Blood pressure control (Buccafusco and Brezenoff 1979)
Lateral	Lateral preoptic	Stress response (Duarte et al. 2017)
	Lateral hypothalamic	Stimulation of food intake and wakefulness
	Supraoptic	Oxytocin production

The paraventricular nucleus is located at the top of the third ventricle (3V). Neurons in the PVN synthesize corticotropin-releasing hormone (CRH), central administration of which reduces food intake and body weight (Dallman 1993). Overproduction of CRH causes anorexia associated with the activation of the hypothalamic-pituitary-adrenal axis (Coste et al. 2001). Oxytocin, which also has anorexigenic effects, is produced in some neurons of the PVN, as well as in the supraoptic nucleus (SON) (Rosen et al. 2008). Oxytocin receptor (OXTR) antagonists can stimulate food intake, and *OXTR*-null mice show increases in meal size during the dark cycle (Blouet et al. 2009, Yamashita et al. 2013).

In contrast, the PVN is also associated with high-fat intake as it contains neurons synthesizing the peptide galanin, which potentiates food consumption with preference for high-fat foods (Akabayashi et al. 1994). Galanin also lowers

insulin secretion during fat absorption, and its expression is enhanced in diabetic rats but suppressed by insulin administration (Akabayashi et al. 1994).

1.1.3. Integration of metabolic state-related signals

The above described neuronal populations respond to hormones, neuropeptides, nutrients, and inputs from other neurons within and outside the hypothalamus (Figure 1.3).

ARC POMC/CART and NPY/AgRP neurons express receptors for leptin, insulin, ghrelin, melanocortins, NPY, and other neuropeptides and neurotransmitters; these signals can modulate electrical activity in the cells to influence the production of peptides (Cheung et al. 1997).

Leptin stimulates the melanocortin system – intraperitoneal administration of leptin increases the level of POMC mRNA in the hypothalamus, while fasting or absence of leptin reduces POMC expression (Schwartz et al. 1997, Mizuno et al. 1998). Moreover, inhibition of MC4R impairs the anorexigenic effects of leptin (Seeley et al. 1997). The expression of another anorexigenic molecule in ARC POMC/CART neurons, CART, is also leptin-dependent (Kristensen et al. 1998).

Additional mechanisms for leptin effects are the inhibition of NPY/AgRP neurons, and increased CRH production in the PVN (Schwartz et al. 1996). NPY signalling is further inhibited by negative feedback from insulin (Elias et al. 1999).

Oestrogen is another modulator of ARC neuronal activity. POMC/CART neurons contain the oestrogen receptor-α (ERα), the expression of which is decreased by fasting in male but not female mice (Iwasa et al. 2017). Mice lacking ERα in POMC/CART neurons exhibit hypometabolism, hyperphagia and abnormal weight gain, particularly in females (Xu et al. 2011).

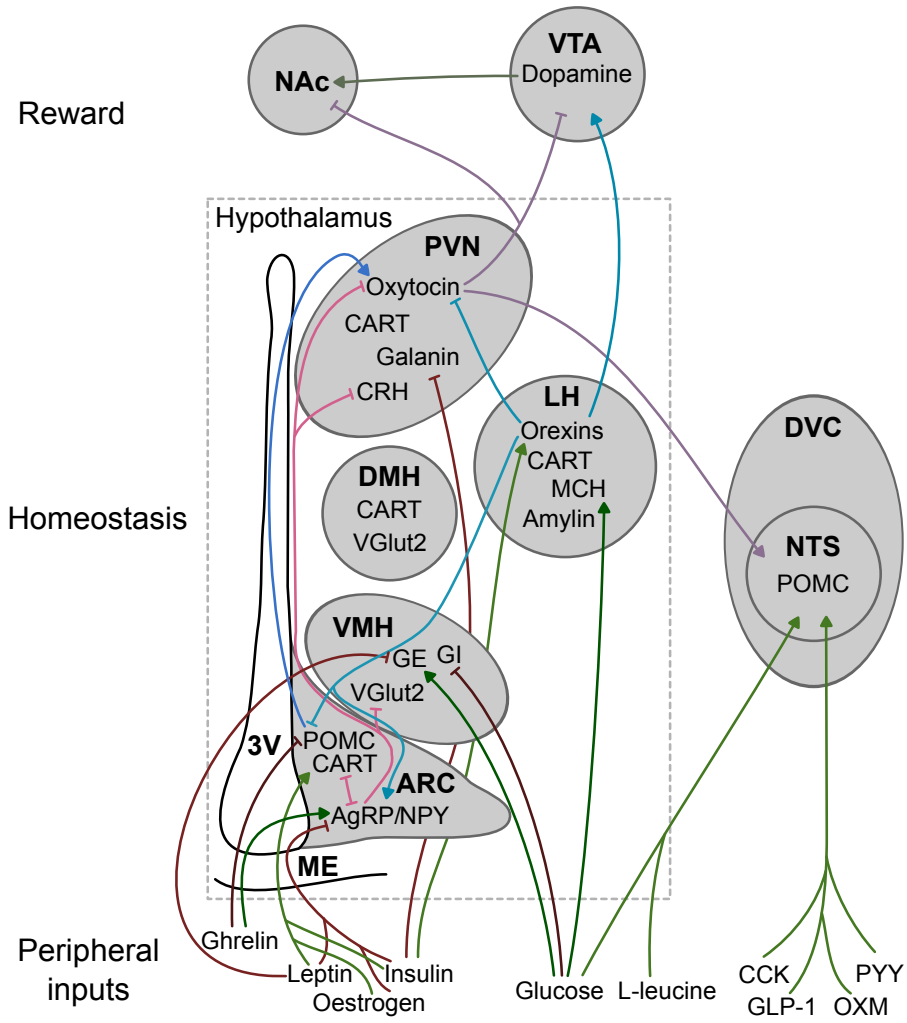

Figure 1.3. Hypothalamic integration of food-related signals. *Orexigenic ghrelin and anorexigenic leptin, insulin and oestrogen access the arcuate nucleus via the median eminence and have opposing effects on POMC/CART and AgRP/NPY neurons. Depending on peripheral inputs, these neuronal populations can inhibit each other as well as modulate firing and neuropeptide production in other nuclei. Insulin also inhibits galanin production, while hypoglycaemia activates orx/hcrt neurons. Glucose activates glucose-excited and inhibits glucose-inhibited neurons in the VMH; in addition, increases in glucose levels induce MCH production in lateral hypothalamus. Glucose, L-leucine and gut peptides also activate NTS POMC neurons. PVN oxytocin neurons are activated by POMC-produced α-MSH and inhibited by NPY, AgRP and orexins; oxytocin activates NTS neurons and inhibits the reward pathway to reduce feeding. Orexins feed back to the arcuate nucleus to inhibit POMC/CART activity and increase the production of AgRP and NPY while activating reward-related dopaminergic neurons. Abbreviations as in Figure 1.2, with the addition of NAc, nucleus accumbens; VTA, ventral tegmental area; DVC, dorsal vagal complex; NTS, nucleus of the solitary tract; CCK, cholecystokinin; GLP-1, glucagon-like peptide 1; OXM, oxyntomodulin; PYY, peptide tyrosine tyrosine.*

ARC POMC/CART and NPY/AgRP neurons project into the PVN, which is rich in MC4 receptors (Mountjoy et al. 1994), as well as anterior hypothalamus, medial preoptic area, LH, DMH, VMH, and posterior hypothalamus (Wang et al. 2015). Both the orexigenic and anorexigenic populations of ARC neurons also activate the sympathetic nervous system in order to influence protein

uncoupling in BAT, thus regulating metabolism by adjusting thermogenesis (Kooijman et al. 2014).

Some LH VGlut2 expressing neurons have been shown to receive inhibitory inputs from the bed nucleus of the stria terminalis in the amygdala, and optogenetic stimulation of these projections produces feeding even in well-fed mice (Jennings et al. 2013).

The PVN contains axons projecting from the NPY/AgRP and POMC/CART neurons of the ARC, and from the orexin neurons of the LH (Elmquist et al. 1998, Elmquist et al. 1999). These inputs determine local release of oxytocin in the PVN, which projects to the dopaminergic neurons of the VTA, POMC neurons of the NTS, and the spinal cord (Sawchenko and Swanson 1982, Shahrokh et al. 2010). Oxytocin receptors are also expressed in other areas of the brain associated with energy homeostasis – VMH, area postrema (AP), the striatum – as well as relevant peripheral tissues, including the gastrointestinal tract and adipocytes (Gimpl and Fahrenholz 2001). The anorexigenic effects of oxytocin have been suggested to be partially due to inhibition of feeding reward circuitry in the nucleus accumbens (NAc) of the striatum as oxytocin administration reduces drug reward behaviour (Carson et al. 2010).

Circadian rhythms form a key regulatory mechanism for feeding behaviour and energy expenditure. The central clock is located in the suprachiasmatic nucleus (SCN), and surgical or genetic manipulations of this nucleus disrupt glucose metabolism (la Fleur et al. 2001), increase food intake and body weight, and decrease energy expenditure (Turek et al. 2005). Peripherally produced hormones leptin and ghrelin have been shown to follow SCN-driven diurnal rhythms (Kalsbeek et al. 2001, Bodosi et al. 2004).

1.1.4. Hypothalamic plasticity

In addition to the intricate mechanisms that rapidly respond to hunger and food consumption, the hypothalamus exhibits a high level of plasticity to provide long-term adaptation to variations in energy availability. The acute responses, i.e. neuronal firing and production of neuropeptides that result in short-term behavioural changes, act to ensure that the bodyweight remains as close as

possible to a set-point around which it constantly fluctuates (Cone 1999). However, chronic exposure to similar conditions, such as a constantly high-fat diet or long periods of fasting, can alter the hypothalamic neural circuits in order to adjust the set-point to the current food environment.

Hypothalamic plasticity occurs on three different levels: changes in synaptic weights; alteration of neuronal properties; and generation of new neurons. The changes in synaptic organisation and cell excitability are quite rapid and can occur within a day (Sternson et al. 2005, Schneeberger et al. 2013), while neurogenesis can take between several days and weeks (Kokoeva et al. 2005, Lee et al. 2014).

Leptin and ghrelin are two of the known regulators of POMC and NPY synaptic plasticity: leptin deficient (*ob/ob*) mice exhibit a lower total number of synapses and a marked increase in the inhibitory tone onto POMC neurons, and an increase in the excitatory tone onto NPY neurons, which can all be reversed to wild-type levels by leptin treatment; furthermore, chronic administration of ghrelin decreases the number of excitatory inputs and reduces the number of inhibitory inputs on POMC neurons without affecting NPY neurons (Pinto et al. 2004). Another study has shown that fasting, which is associated with a high level of ghrelin, also reduces the strength of excitatory inputs on ARC POMC neurons from the VMH, and the effect is not rescued by leptin (Sternson et al. 2005). An overnight fast is enough to form new excitatory synapses on orx/hcrt neurons (Horvath and Gao 2005).

Oestrogen, like leptin, robustly increases the number of excitatory inputs on POMC neurons independently of leptin receptor signalling (Gao et al. 2007). A high-fat diet has been found to increase hypothalamic expression of polysialic acid, which modulates distances between cells thus promoting cellular reorganisation, and to increase the frequency of miniature excitatory postsynaptic currents in POMC neurons (Benani et al. 2012). In seasonal mammals, plasticity is observed in GnRH neurons, while annual changes in thyroid hormone availability regulate genes involved in synapse formation (Jansen et al. 2003, Zhang et al. 2008).

The activation of POMC neurons depends on mitochondrial fusion, while the development of these cells requires mitochondrial fission – processes during which mitochondria fuse or divide to adapt their number and morphology to the environmental conditions (Schneeberger et al. 2013). Faults in these processes are associated with type 2 diabetes and obesity (Schneeberger et al. 2013, Toda et al. 2016). The deletion of the mitochondrial fission regulator dynamin-related protein 1 (DRP1) in mature POMC neurons results in higher leptin and glucose sensitivity (Santoro et al. 2017). Additionally, fasting increases DRP1 expression to reduce POMC excitability, thus silencing the anorexigenic circuit in ARC and promoting feeding (Santoro et al. 2017).

The hypothalamus has been recently discovered to form one of the adult neurogenic niches (Pencea et al. 2001). Hypothalamic neurogenesis has been demonstrated to function as a compensatory mechanism for chronic AgRP neurodegeneration, allowing mice to maintain normal levels of food intake despite the disruption in the ARC orexigenic circuit (Pierce and Xu 2010). In contrast, ciliary neurotrophic factor (CNTF), used as a drug to treat obesity, induces cell proliferation in the hypothalamus to produce leptin-sensitive neurons which in turn promote long-term weight loss (Kokoeva et al. 2005). Moreover, the generation of new neurons in this area is diet-responsive: high-fat diet (HFD) disrupts hypothalamic neurogenesis in the parenchyma of the mediobasal hypothalamus (MBH), which results in weight gain and obesity (Li et al. 2012); conversely, both HFD and low-protein diet can increase neural progenitor cell proliferation in the ME of female rats (Lee et al. 2014).

The cell types that have been found to proliferate into new neurons in the hypothalamus are NG2 glia, also known as oligodendrocyte precursor cells (Robins et al. 2013), and tanycytes (Haan et al. 2013, Robins et al. 2013). Hypothalamic tanycytes can differentiate into both glial cells and neurons (Xu et al. 2005).

The complexity of the adaptive mechanisms in the hypothalamus required to maintain bodyweight reflects the importance of these processes for animal survival. From an evolutionary perspective, most animals have always gone through long periods of scarce food availability; the processes of tuning down

the anorexigenic mechanism and increasing the activation of orexigenic circuits therefore function to maintain motivation and drive to feed throughout these periods, while hypothalamic changes in the opposite direction are required when food is abundant to avoid the negative effects of overeating such as inflammation and obesity (Zeltser et al. 2012).

1.1.5. Other neuronal mechanisms of food intake control

The dorsal vagal complex (DVC) in the hindbrain is linked to the control of meal size. Chronic administration of oxytocin activates neurons in this area, as well as the hypothalamus and amygdala, which can lead to weight loss in obese rodents due to reduced meal sizes, increased latency of the first meal and increased energy expenditure (Arletti et al. 1990, Takayanagi et al. 2008).

Nucleus of the solitary tract is an important centre of convergence and integration of signals related to energy availability and metabolic state (Blouet and Schwartz 2012). The NTS receives direct input from the gastrointestinal tract and can negatively control food intake (Blevins et al. 2000). Just like the ARC, VMH and PVN in the hypothalamus, the NTS is in close proximity to a circumventricular organ, the area postrema, allowing a range of circulating factors to reach the neurons in this nucleus (Borison 1974). NTS neurons have a role in glucose sensing and the response to hypoglycaemia (Ritter et al. 2000), and can detect L-leucine (Blouet and Schwartz 2012).

POMC is also expressed in some neurons in the brainstem, mostly in the AP and the NTS (Padilla et al. 2011). A study in *Pomc-Cre* mice has revealed that brainstem POMC neurons are involved in acute suppression of feeding, while hypothalamic POMC neurons regulate long-term feeding behaviour, as the onset of the effects of ARC POMC neurons is slow (Zhan et al. 2013).

The VTA is a part of the mesolimbic reward pathway with dopaminergic neurons projecting into the NAc (Kelley and Berridge 2002). Neurons in the VTA detect leptin, GLP-1 and ghrelin (Naleid et al. 2005, Hommel et al. 2006, Dickson et al. 2012), and are activated by orexins (Korotkova et al. 2003), forming a close link between homeostatic and hedonic regulation of feeding.

Some neurons in the striatum, including those in NAc, express the orphan G-protein coupled receptor GPR88 (Van Waes et al. 2011). *Gpr88*-KO mice exhibit increased lean mass and fat loss on HFD, as well as decreased food intake and energy expenditure on standard chow; the changes in feeding and metabolism in these mice are attributed to *Cart* upregulation in ARC and downregulation in DMH and LH (Lau et al. 2017).

New mechanisms and factors involved in the regulation of energy homeostasis are constantly emerging; the central and peripheral factors described so far in this thesis are only the ones that are already widely recognised, but ongoing research will undoubtedly continue to provide an increasingly detailed picture of food intake and energy expenditure control.

1.1.6. Glia and energy homeostasis

Originally, most research on central regulation of energy homeostasis focussed on neurons; in recent years, non-neuronal cells have also received substantial attention, revealing their roles in the control of food intake and energy expenditure in both health and disease.

The major glial cell types found in the hypothalamus are astrocytes, microglia, NG2 glia, and the ependymal layer of 3V, formed of ependymal cells and tanycytes. Astrocytes are best known for their roles in synapse formation, maintenance, and the modulation of synaptic transmission (Chung et al. 2015). Microglia act as immune cells in the brain (Gehrmann et al. 1995). NG2 glia are precursors to oligodendrocytes – cells involved in neuron myelination (Baumann and Pham-Dinh 2001) – and, to some extent, new neurons (Robins et al. 2013). The ependymal layer of the 3V regulates the exchange of hormones, nutrients and other substances between the CSF and hypothalamic parenchyma (Langlet 2014). A more diverse set of functions, most of which relate to metabolism or reproduction, have been attributed to hypothalamic tanycytes in recent years and will be discussed further in following sections.

Obesity leads to increases in the production of pro-inflammatory markers in the hypothalamus (De Souza et al. 2005). Even short-term (under 1 week) intake of HFD is associated with hypothalamic injury, the hallmarks of which are

autophagy and microglial accumulation, or microgliosis, in the ARC (Thaler et al. 2012). A distinction has been made between obesity caused by hormonal imbalance and HFD: increased microglial activation in ARC is observed only under HFD conditions but not in leptin deficient animals, meaning that diet, but not obesity alone, induces hypothalamic inflammation (Gao et al. 2014). Peripheral signals such as leptin and GLP-1 can regulate the level of microglial activation as microglia have recently been shown to express the leptin receptor, and mice with a specific deletion of leptin receptor from microglia and macrophages have increased food intake and bodyweight (Gao et al. 2014, Gao et al. 2017).

While the microglia-driven inflammation is commonly recognised as only a consequence of high-fat feeding, new evidence shows that microglia are actually critical regulators of susceptibility to diet-induced obesity (DIO), as microglial activation is required for both the onset of hypothalamic inflammatory processes and the control of energy balance in response to HFD (Valdearcos et al. 2017). The level of inflammation in the hypothalamus after chronic HFD intake correlates with the number of microglial cells present in ARC and ME; and depletion of microglia in the area restores leptin signalling, which is otherwise impaired by prolonged exposure to saturated fatty acids (Valdearcos et al. 2014).

Interestingly, ARC astrogliosis also occurs rapidly in response to HFD, but the number of astrocytes returns to baseline level over several weeks (Thaler et al. 2012). However, if increased fat intake persists, astrocytes begin to accumulate again (Thaler et al. 2012).

Astrogliosis has been shown to have a protective effect against HFD: astrocytic nuclear factor kappa B (NFκB) prevents HFD-induced hyperphagia, while a disruption the NFκB transcription pathway increases caloric intake in the short term (Buckman et al. 2013).

While microgliosis is mostly induced by DIO, astrogliosis is also observed in a genetic model of obesity, the MC4 receptor deficient mouse (Buckman et al. 2013). Astrogliosis also differs from microgliosis in its spatial organisation as

GFAP immunoreactivity shows pronounced astrogliosis in response to HFD in most hypothalamic nuclei, with the highest increases in ARC and DMH but only small changes in VMH, LH and anterior hypothalamic nucleus (Buckman et al. 2013).

Besides neuroinflammation, astrocytes are involved in the plasticity of neuronal populations that control energy homeostasis, as increased glial ensheathment of POMC neuron cell bodies is associated with loss of synapses (Horvath et al. 2010). Some astrocytes also express receptors for leptin, insulin, and melanocortins, and astrocyte-specific leptin signalling has been shown to affect neural plasticity in ARC (Kim et al. 2014). Experiments involving chemogenetic astrocyte activation showed that astrocytes could potentiate anorexia induced by direct leptin administration and reduce hyperphagia caused by a ghrelin injection via an adenosine-dependent pathway (Yang et al. 2015); however, a later study challenged these findings showing that astrocyte activation stimulates both ARC NPY/AgRP and POMC/CART neurons, but the strong inhibitory signal from NPY/AgRP to POMC/CART neurons suppresses POMC/CART activity, producing an overall orexigenic effect (Chen et al. 2016). The conflicting results may be attributed to drug dosage, as the former study used a high dose of clozapine-N-oxide which was later suggested to have non-specific effects (Chen et al. 2016).

Astrocytes are responsible for most of brain fatty acid metabolism into ketone bodies, used by neurons as an energy source in low glucose conditions (Blázquez et al. 1999). VMH astrocyte ketone body production can override neuronal glucose and fatty acid sensing and reduce feeding following ingestion of a high-fat meal (Le Foll et al. 2014).

Another type of glial cell, originally known as oligodendrocyte precursor cells, termed NG2 glia, have a role in the regulation of energy expenditure, as mice lacking the *NG2* gene in these cells exhibit a lean phenotype, possibly due to the hypothalamic hypomyelination resulting from a lower number of oligodendrocytes (Chang et al. 2012). More recent evidence suggests that a small proportion of NG2 glia can also give rise to hypothalamic neurons, which could further explain the involvement of these cells in metabolic control (Robins

et al. 2013). Moreover, NG2 glia can respond to glutamate and ATP with increases in intracellular Ca^{2+}, which is potentially involved in glial scarring after injury (Butt et al. 2005).

1.2. Hypothalamic tanycytes

The term “tanycytes” – Greek for “elongated cells” – was first introduced by Ernst Horstmann in 1954, when he established in a histological study that the shark brain ependyma consisted of multiple cell types (Horstmann 1954). Hypothalamic tanycytes are non-neuronal, radial glia-like cells that line the walls and the floor of the third ventricle (Bolborea and Dale 2013). They lie next to ependymal cells and are sometimes considered to be a type of ependymal cell, although there are two major differences: instead of cilia, tanycytes have microvilli on their apical surface, and their long processes can exceed 200 µm into the brain parenchyma.

Based on location, there are four subtypes of tanycytes: α1, α2, β1, β2, while some researchers have also described γ tanycytes (Wittmann et al. 2017). α tanycytes are located in the dorsal region of 3V, with α1 tanycytes interspersed by ependymal cells, and project into the ARC (Brawer 1972, Bruni et al. 1985) and the VMH (Rodríguez et al. 2005). β1 tanycytes occupy the ventral part of the wall of 3V, while β2 tanycytes line the floor or the ventricle. The processes of β tanycytes project into the external region of the ME. Tanycytes with cell bodies located within the ME or the pituitary stalk are referred to as γ tanycytes (Wittmann et al. 2017).

1.2.1. Morphology and physiology

Tanycyte cell bodies are elliptical, about 15 µm long, with a basal prolongation of up to 200 µm, and microvilli on the apical surface contacting the CSF of the 3V. Interestingly, most tanycyte nuclei are also ovoid in shape, while the nuclei of ependymal cells are round (Jarvis and Andrew 1988). There is substantial evidence of gap junctions between adjacent tanycytes, with the number of junctions ranging from one single channel to aggregates of over 2000 junctions (Jarvis and Andrew 1988).

Tanycyte resting potential is around -80 mV, and they do not exhibit spontaneous action potentials, spontaneous postsynaptic potentials, or any other changes in membrane potentials over time. Their input resistance is very low (≪ 1 MΩ) compared to other glial cells in the area (2–20 MΩ) (Jarvis and Andrew 1988). This is likely to be a consequence of extensive electrical coupling via gap junctions.

Tanycytes express a range of cell markers, including the glial marker GFAP (Gould et al. 1990). The expression of these markers is location-dependent and some can be limited to β tanycytes (e.g. FGF-1, FGF-2, CNTFR); others can be expressed in both α and β tanycytes (e.g. nestin, GPR50, Rax, DIO2), or α tanycytes and ependymal cells (e.g. GLAST, CNTF, Cx43); three markers are known to be expressed in all tanycytes and ependymal cells: Sox2, vimentin and GLUT1 (Goodman and Hajihosseini 2015). Such distribution of marker expression indicates that tanycytes are a highly heterogeneous cell population.

1.2.2. Functions

Initial research on hypothalamic tanycytes in the 1980s provided evidence for the involvement of these cells in mechanical support of the third ventricle, phagocytosis, regulation of extracellular space constituents and transport between cerebrospinal fluid and the portal vasculature (Bruni et al. 1985, Jarvis and Andrew 1988). Tanycytes have also long been known for their high ATPase activity, which was linked to their transport functions (Firth and Bock 1976).

More recent interest in tanycytes has revealed that they also serve a range of different purposes, such as regulation of the secretory activity of neuroendocrine neurons, including β tanycytes being involved in the control of blood-hypothalamus barrier permeability (Mullier et al. 2010) and the GnRH regulatory system (Ojeda et al. 2008). Tanycytes of the ME are indirectly responsive to oestrogen (the mechanism requires NO release from endothelial cells), which induces an acute and reversible retraction of tanycyte processes to increase neurovascular contacts between GnRH neurons and ME capillaries (De Seranno et al. 2010). Tanycytes also participate in thyroid hormone metabolism as they express type 2 deiodinase, allowing them to take up thyroxine (T4) from CSF and convert it to triiodothyronine (T3) before

transporting it to neurons via a specific thyroid hormone transporter MCT8 (Kalló et al. 2012).

Another recently discovered tanycyte function is their role as adult neuronal stem cells as tanycytes can self-renew and give rise to neurons (Sousa-Ferreira et al. 2014). α tanycytes express fibroblast growth factors (FGF) 10 and 18, and FGF-2 signalling induces cell proliferation (Robins et al. 2013). FGF-10-expressing β tanycytes have been shown to generate parenchymal neurons, primarily in ARC and VMH, but also in DMH and LH, and glial cells, suggesting a major role for tanycyte neurogenesis in the control of energy homeostasis (Haan et al. 2013). Moreover, HFD or a low-protein diet (LPD) can reduce neurogenesis in ARC, but HFD also selectively enhances neurogenesis in ME in females (Lee et al. 2012, Lee et al. 2014).

Tanycytes also have chemosensitive properties, including glucosensing and fatty acid metabolism and transport, which may have some influence on their stem cell activity (Dale 2011, Hofmann et al. 2017). Tanycyte glucose sensitivity was originally proposed to occur via glucose transporter 2 (GLUT2) and K_{ATP} channels (García et al. 2003), but was later also found to be sweet taste receptor-dependent (Benford et al. 2017); both pathways are suspected to influence feeding (Benford et al. 2017, Barahona et al. 2018). Tanycyte fatty acid metabolism is altered in obesity (Hofmann et al. 2017).

1.2.3. Potential role in food intake control

In addition to the aforementioned functions, tanycytes might be a key player in energy homeostasis. Their position in the walls and the floor of the third ventricle means that they have privileged access to the CSF. Tanycyte processes extend into the hypothalamic nuclei that regulate food intake and energy expenditure; the projections come into close contact with neurons within those nuclei, which suggests that communication is possible between tanycytes and hypothalamic neurons (Bolborea and Dale 2013). Paired with their chemosensitivity and ability to signal via Ca^{2+} and ATP (Benford et al. 2017), the potential tanycyte-to-neuron communication could be established as a new mechanism of monitoring nutrient availability in the hypothalamus (Bolborea

and Dale 2013). Emerging evidence shows that tanycyte expression of the glucose transporter GLUT2 and glucokinase have a role in food intake regulation of fasted rats, and the mechanism involves tanycyte lactate signalling to POMC neurons (Uranga et al. 2017, Barahona et al. 2018). However, GLUT2 is also involved in cell metabolism, thus its deletion in tanycytes could affect food intake by reducing tanycyte viability rather than directly inhibiting glucose-related tanycyte signalling to ARC neurons.

ME tanycytes form a gateway for ghrelin (Collden et al. 2015) and leptin into MBH, and diet-induced obesity impairs tanycyte leptin transport (Balland et al. 2014). Conversely, fasting increases the permeability of the tanycyte layer to blood-borne molecules (Langlet et al. 2013). Some rat tanycytes express the *Pomc* gene, which is responsible for neuropeptides that regulate energy homeostasis, and was until recently thought to be expressed exclusively by neurons (Wittmann et al. 2017). Mouse tanycytes produce CNTF, the administration of which reduces food intake in obese rodents and counteracts positive energy balance (Severi et al. 2013). The receptor for interleukin-6, a pro-inflammatory cytokine known to negatively influence food intake and energy balance, similar in its actions and detection pathway to leptin, has recently been localised to tanycytes (Anesten et al. 2017).

Tanycytes are also increasingly known as diet-responsive neural stem cells. Lineage tracing studies have demonstrated that tanycytes can generate new neurons and glia, meaning that the neuronal networks of the hypothalamus are highly plastic and can be remodelled by diet (Haan et al. 2013).

1.3. Amino acid sensing in the brain

Protein is the most effective type of macronutrient for satisfying hunger and providing a long period of satiety (Mellinkoff et al. 1956). This is partly because of the slower digestion of protein-rich foods, as well as the fact that such foods keep blood glucose levels relatively constant, therefore reducing potential food cravings that could otherwise occur soon after a meal. As well as their satiating effect, high-protein diets are known to reduce body fat (Weigle et al. 2005); however, increasing dietary protein content to 50% and beyond can have

adverse consequences such as nephromegaly and impaired calcium homeostasis (Barzel and Massey 1998, Hammond and Janes 1998). Diets containing 70% or more protein are not palatable, causing taste aversion and food rejection (Tews et al. 1992).

It is becoming increasingly evident that amino acids derived from dietary protein mediate their satiating effect via the brain. Direct ICV injections of selected essential amino acids into the hypothalamus, thus bypassing the digestive system, suppressed appetite in rats (Panksepp and Booth 1971, Cota et al. 2006). Better understanding of brain amino acid detection and the related satiating effects could therefore be an essential step in further unravelling the mechanisms behind the regulation of energy metabolism.

1.3.1. Amino acid transport into the brain

After a meal, the concentrations of dietary amino acids increase in both blood plasma (Frame 1958) and CSF (Glaeser et al. 1983). Interestingly, these rises are not necessarily reflective of the amino acid contents of the food, as some amino acids exhibit larger fluctuations in the blood than others (Frame 1958). Moreover, brain uptake of dietary amino acids from the blood also varies (Glaeser et al. 1983).

Amino acids from blood plasma can cross the blood-brain barrier in the region of the median eminence, which has increased permeability, to enter the hypothalamus (Sclafani et al. 2014). There are several different transporter systems that take the blood plasma amino acids across the blood-brain barrier: System L for neutral amino acids with large side chains; System y^+ for sodium-independent uptake of cationic amino acids; System x^- for anionic amino acids; System N for sodium-dependent uptake of L-glutamine (Gln), L-histidine (His) and L-asparagine (Asn); Systems A and ASC for small neutral amino acids; System B^{o+} for neutral and basic amino acids; and System X^- for L-glutamate (Glu) and L-aspartate (Asp) (Oldendorf and Szabo 1976, Smith 2000). Amino acid transporters are upregulated in hypothalamic cells during amino acid starvation (Hellsten et al. 2017).

The baseline levels of amino acids in rat CSF vary from 1–5 μM for isoleucine (Ile), proline (Pro), tryptophan (Trp), cysteine (Cys) and Asp to up to 500 μM for glycine (Gly) (Okame et al. 2015). Some amino acids make particularly effective signals of food intake – for example, the concentrations of L-tyrosine (Tyr), L-methionine (Met), Pro, and L-alanine (Ala) markedly increase in the brain within 2 hours of meal consumption (Glaeser et al. 1983). Others, such as L-valine (Val), L-leucine (Leu), L-lysine (Lys) and His, only increase if the ingested food is high in protein, thus representing the nutritive quality of the meal (Glaeser et al. 1983): for example, a high protein meal can induce a twofold increase in plasma Leu and a 24% increase in CSF Leu (Blouet et al. 2009). Microdialysis experiments in freely moving rats have shown an increase in Ala, Ile, Leu, Met, threonine (Thr), Tyr and Val concentrations in the lateral hypothalamic area within 20–40 minutes of dietary amino acid gavage (Choi et al. 1999). CSF concentrations of all these amino acids plus Asn and Gly in the medial preoptic area reflect dietary protein content (Choi et al. 2000).

1.3.2. Brain amino acid detection mechanisms

There are several neuronal mechanisms of direct amino acid sensing that have already been described in the brain. Extensive work by the Gietzen laboratory has shown that the mechanisms for brain amino acid sensing are so specific that injecting one essential amino acid reverses the negative effect of a diet lacking that particular amino acid (Gietzen and Magrum 2001).

Some neurons (around 90% of NPY/AgRP and 45% of POMC) in the mediobasal hypothalamus express the mammalian target of rapamycin (mTOR), which is indirectly activated by amino acids and has been described as an ATP sensor (Dennis et al. 2001, Cota et al. 2006). Hypothalamic expression of SLC38A9, an amino acid transporter associated with mTOR activation, increases after exposure to high-fat diet (Hellsten et al. 2017). The umami taste receptor Tas1r1/Tas1r3 has been shown to facilitate amino acid detection in the mTOR pathway (Wauson et al. 2012). The local metabolism of ICV administered Leu, a potent activator of mTOR, reduces the levels of NPY in ARC, produces anorexia and causes weight loss without affecting oxygen consumption, respiratory rate, core temperature or activity (Cota et al. 2006,

Blouet et al. 2009). Val, similar in structure but unable to activate mTOR, does not have such an effect (Cota et al. 2006).

In contrast to central administration, chronic oral Leu supplementation has no anorectic effect, despite being sensed by neurons (Zampieri et al. 2013). Moreover, additional, mTOR-independent Leu activation of MBH neurons has recently been demonstrated, casting doubt on the widely accepted hypothalamic mTOR significance (Heeley et al. 2018).

Nevertheless, mTOR signalling is also known to be used for amino acid sensing in the caudomedial part of the NTS, which integrates signals from the gastrointestinal tract and has influence on food intake (Blouet and Schwartz 2012). Evidence of immunoreactivity of the mTOR downstream target pS6 has been found in NTS POMC and CCK neurons; inhibition of another mTOR downstream molecule, a nutrient sensing enzyme S6K1, rapidly increases meal size and latency (Blouet and Schwartz 2012).

The anterior piriform cortex (APC) has been suggested as the site of recognition of amino acid imbalanced diets (Leung and Rogers 1971). Hao and colleagues have found evidence that the accumulation of uncharged tRNA is the main signal of essential amino acid deficiency in the APC (Hao et al. 2005).

Orx/hcrt neurons of the LH, involved in feeding and sleep behaviours and speculated to link hypothalamic and cortical networks, are sensitive to non-essential amino acids and respond to a dietary mix of amino acids using a mechanism that involves K_{ATP} and System A amino acid transporters (Karnani et al. 2011).

1.4. Amino acid receptors

The amino acid detection mechanisms described in the previous section mostly involve complex cascades of events in order to activate the cells. In addition to these systems, there are receptors for direct detection of amino acids, which are expressed in mammals throughout the organism.

Most known amino acid sensitive receptors are a part of the class C G protein-coupled receptor (GPCR) family. A summary of GPCR amino acid detection

pathways is shown in Figure 1.4. One of these receptors, G protein-coupled receptor class C group 6 member A (GPRC6A), when expressed in *Xenopus* oocytes, responds to L-amino acids arginine, lysine, alanine, serine, ornithine, and citrulline (Wellendorph et al. 2005). It is also sensitive to Ca^{2+}, other cations, and osteocalcin (Christiansen et al. 2007), and it may be involved in some effects of steroid hormones (Pi et al. 2010). Human tissue analysis showed GPRC6A mRNA expression in a wide range of tissues, which was particularly strong in the brain, skeletal muscle, leukocytes and testes, medium in liver, heart, kidney and spleen, but not detectable in the tongue, small intestine, or colon (Wellendorph and Bräuner-Osborne 2004). Some GPRC6A-null mice exhibit insulin resistance, hyperglycaemia, and decreased bone mineral density (Pi et al. 2008). However, GPRC6A is not associated with high protein diet-induced weight loss (Kinsey-Jones et al. 2015).

Another receptor in this family, the calcium-sensing receptor (CaSR), is sensitive to extracellular Ca^{2+}, other divalent and trivalent cations and pH, and is potentiated by L-amino acids, with preference for aromatic over polar amino acids as allosteric modulators (Conigrave et al. 2000). CaSR is expressed in gastric, intestinal and pancreatic tissues (Brown 1999).

Two amino acids are major neurotransmitters: L-glutamate is excitatory and γ-aminobutyric acid (GABA) is inhibitory; specialised receptors for them are expressed in neurons and astrocytes throughout the brain. The GABA receptors $GABA_A$ and $GABA_B$, as well as the ionotropic glutamate receptors (iGluRs) known as the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, the N-methyl-D-aspartate (NMDA) receptor, and the kainate (KA) receptor, detect these amino acids as signalling molecules (Sivilotti and Nistri 1991, Dingledine et al. 1999); on the other hand, some metabotropic glutamate receptors (mGluRs) also function as taste receptors for L-glutamate and several other amino acids (Chaudhari et al. 1996).

Amino acids have distinct flavours, which allows animals to detect them in food. Most D-amino acids are sweet; some L-amino acids, such as L-tryptophan and L-phenylalanine, are bitter; the flavours of most non-aromatic L-amino acids are

species-specific (Toda et al. 2013). The following section focuses on amino acids known to have an umami flavour.

1.4.1. Umami taste receptors

Umami is a savoury taste produced in humans by L-glutamate (often present in food as the flavour enhancing additive monosodium glutamate) and L-aspartate, best described as the meaty taste of broth. In rodents, most non-aromatic amino acids are considered to be umami tastants. The umami taste has been long defined by its potentiation by purine nucleotides such as inosine 5'-monophosphate (IMP) and sensitivity to the mGluR agonist L-AP4 (Kurihara and Kashiwayanagi 1998). As a likely candidate for an umami taste receptor based on these properties, Nelson *et al* suggested Tas1r1/Tas1r3, a taste receptor expressed in the tongue, very similar to the sweet taste receptor Tas1r2/Tas1r3 (Nelson et al. 2002).

Both sweet and umami Tas1 taste receptors are also class C GPCRs. They are both heterodimeric, sharing the Tas1r3 subunit. The Tas1r1 and Tas1r2 subunits have not been found to form homodimers or function by themselves; Tas1r3 has been shown to function as a human and mouse receptor for the taste of calcium and a mouse receptor for magnesium (Tordoff et al. 2012). The sweet taste receptor Tas1r2/Tas1r3 is responsive to various sweet stimuli: glucose, fructose, sucrose, maltose, various sweet proteins and synthetic sweeteners such as acesulfame K, aspartame, saccharin and others (Li et al. 2002). The umami taste receptor Tas1r1/Tas1r3 is known to function as a broadly tuned amino acid receptor, highly selective for most L-amino acids (Nelson et al. 2002), although the ligand specificity is species-dependent (Toda et al. 2013). While the human variant of the receptor exhibits the greatest sensitivity to L-glutamate and L-aspartate, there is evidence of human embryonic kidney cells that express Tas1r1/Tas1r3 responding, albeit weakly, to L-alanine, L-serine, L-glutamine, L-asparagine, L-arginine and L-histidine (although according to the authors of the study, the responses to L-arginine and L-histidine may have been artefacts caused by high osmolarity of the solutions used in the experiment) (Toda et al. 2013). The mouse Tas1r1/Tas1r3 is

significantly less sensitive to acidic amino acids but has greater sensitivity to other non-aromatic amino acids (Toda et al. 2013).

Tas1r1/Tas1r3 possesses a large extracellular Venus flytrap domain linked to a smaller cysteine-rich domain and a seven-transmembrane domain (Cascales et al. 2010). The binding site for the amino acids is located on the Tas1r1 subunit, in the hinge region of the Venus flytrap domain, while the IMP-binding site lies near its opening (Zhang et al. 2008).

After the binding of an amino acid to the Tas1r1 subunit, the G protein gustducin stimulates phospholipase C β2 (PLC-β2), which hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP2) into inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol. IP_3 then induces Ca^{2+} release, which leads to the gating of the cation channel transient receptor potential cation channel subfamily M member 5 (TRPM5; Figure 1.4) (Chandrashekar et al. 2006). TRPM5 opens large membrane channels, which allow ATP release from the cell (Huang and Roper 2010).

Other candidates for umami taste detection are brain-expressed and taste-expressed variants of type 1 and 4 mGluRs (mGluR1 and mGluR4) (Chaudhari et al. 2000, Maruyama et al. 2006). The brain variants of these receptors are expressed in a subset of cells on both the anterior and posterior tongue in rats, while the taste variants are located on the posterior tongue and have a truncated amino-terminal domain (San Gabriel et al. 2005). The stimulation of mGluRs activates a cyclic adenosine monophosphate (cAMP)-dependent pathway, leading to an increase in intracellular Ca^{2+} to open membrane channels for ATP release. It has recently been shown that mice lacking the Tas1r3 subunit of the Tas1r1/Tas1r3 receptor, or its downstream target TRPM5, can still taste umami substances to some extent, but their sensitivity can be further reduced by a group I mGluR antagonist (*RS*)-1-aminoindan-1,5-dicarboxylic acid (AIDA) for mGluR1, or group III mGluR antagonist (*RS*)-α-cyclo- propyl-4-phosphonophenylglycine (CPPG) for mGluR4 (Yasumatsu et al. 2012). Thus, there are at least three different receptors in the tongue for amino acid detection.

While mGluRs are primarily activated by glutamate, new evidence suggests that different L-amino acids such as L-arginine and L-serine can induce responses in isolated mouse taste cells (Choudhuri et al. 2016).

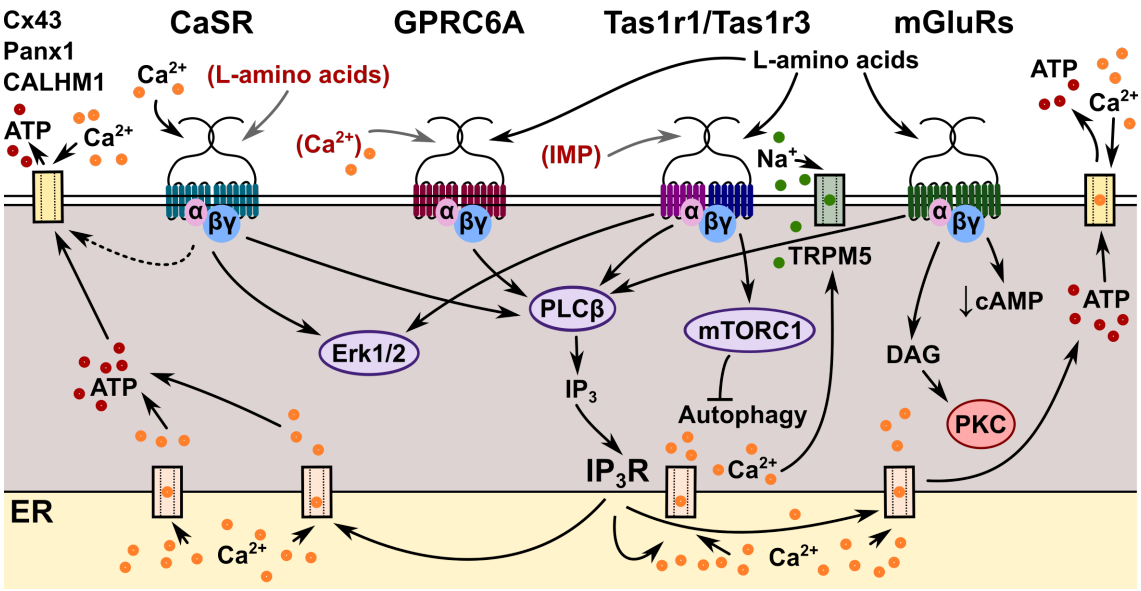

Figure 1.4. Amino acid-sensitive G protein-coupled receptor pathways. *Calcium-sensing receptor (CaSR) is activated by Ca^{2+} and potentiated by L-amino acids. The binding of Ca^{2+} and amino acids to the receptor activates G proteins α and βγ, which leads to ERK1/2 phosphorylation, activation of PLC-β2, and, to a lower extent, direct opening of large conductance membrane channels that facilitate the uptake of extracellular Ca^{2+} and the release of ATP. PLC-β2 triggers Ca^{2+} release from the intracellular stores via IP_3 receptors; the increase in intracellular Ca^{2+} then leads to ATP production and release. GPRC6A is activated by L-amino acids and modulated by Ca^{2+}, and its activation also initiates the PLC-β2 pathway. Tas1r1/Tas1r3, sensitive to most non-aromatic amino acids and positively modulated by IMP, follows the same pathways as the former two receptors, but the increase in intracellular Ca^{2+} also opens TRPM5 to allow Na^{+} influx. Tas1r1/Tas1r3 also activates mTOR complex 1 to inhibit autophagy. mGluRs signal by activating PLC-β2 or protein kinase C, or decreasing cAMP. CaSR, calcium-sensing receptor; Cx43, connexin 43; panx1, pannexin 1; CALHM1, calcium homeostasis modulator 1; ERK1/2, extracellular signal-regulated protein kinases 1&2; PLC-β2, phospholipase C β2; IP_3, inositol-1,4,5-trisphosphate; mTORC1, mammalian target of rapamycin complex 1; TRPM5, transient receptor potential cation channel subfamily M member 5; DAG, diacylglycerol; PKC, protein kinase C; cAMP, cyclic adenosine monophosphate; ER, endoplasmic reticulum.*

Among the three described amino acid receptors, Tas1r1/Tas1r3 is the only one to show synergistic responses to amino acids and nucleoside monophosphates (Damak et al. 2003). IMP is a compound of both human and rodent umami taste. It is known to enhance the umami taste properties in a concentration-dependent manner (in the range of 0.1–10 mM) by stabilizing the closed conformation of the Venus flytrap domain of the Tas1r1 subunit once an amino acid has attached to it (Zhang et al. 2008, Yasumatsu et al. 2012). Most studies show that IMP alone does not elicit a response in cells expressing Tas1r1/Tas1r3 (but responses to IMP were observed in *Tas1r3*-null cells in work focusing on mGluRs – see Pal Choudhuri *et al.*, 2016), and it does not affect sweet taste sensing (Nelson et al. 2002).

As most D-amino acids have a sweet flavour, they are most likely detected by the sweet taste receptor. The several bitter L-amino acids such as L-phenylalanine and L-tryptophan activate bitter taste receptors of the Tas2 receptor family, as well as the CaSR (Conigrave et al. 2000, Bassoli et al. 2014).

1.4.2. ATP signalling

Glial and taste cells cannot fire action potentials to communicate among themselves or with neurons; instead, they use Ca^{2+} signalling and neuroactive chemicals termed gliotransmitters. Some of the currently known gliotransmitters are ATP and adenosine, glutamate, and D-serine (Parpura et al. 1994, Haydon and Carmignoto 2006).

Glial Ca^{2+} signalling is dependent on GPCR activation and is used for glia-to-glia communication via gap junctions as well as to trigger gliotransmitter release that can activate both surrounding glial cells and neurons (Fiacco and McCarthy 2006).

ATP is well established as a signalling molecule that mediates various biological effects via purine receptors. It can be released into the extracellular space either in vesicles, or via membrane channels (Bodin and Burnstock 2001). Neighbouring cells that express P2 receptors on the cell surface then pick up the signal (Ralevic and Burnstock 1998).

Several large, non-selective membrane channels are permeable to ATP. Taste receptor cells have been shown to secrete ATP mainly via gap junction hemichannels composed of connexins or pannexins, and via calcium homeostasis modulator 1 (CalHM1).

Pannexins are a class of gap junction-like proteins, one of which – pannexin1 (panx1) – forms hemichannels in astrocytes for ATP release (Iglesias et al. 2009). Panx1 has been demonstrated to mediate the release of ATP from taste receptor cells in both isolated cell preparations (Huang et al. 2007) and intact taste buds in tongue slices (Dando and Roper 2009).

Connexins are vertebrate gap junction proteins. They form gap junctions in glial cells, allowing direct cell-to-cell communication (Stout et al. 2002). Connexin 43 (Cx43) has been identified in tanycytes of the 3V and is likely to contribute to tanycyte control of the brain-cerebrospinal fluid barrier permeability between the fenestrated capillaries of the ME and the 3V, as well as the release of small molecules from tanycytes to hypothalamic neurons (Szilvásy-Szabó et al. 2017). Connexin hemichannels have also been suggested as part of the taste-induced ATP signalling system in the tongue (Romanov et al. 2007). However, the evidence in favour of connexin hemichannels being used for ATP release from taste receptor cells has been challenged by several authors: firstly, many studies on connexin channels have used carbenoxolone (CBX), which has a much higher potency for pannexins (by an order of magnitude); low concentrations of CBX are as effective as high concentrations at blocking ATP release from taste cells (Huang et al. 2007). Secondly, there has been a suggestion that an increase of intracellular Ca^{2+}, observed after the activation of taste receptors, can actually close connexin hemichannels (Li et al. 1996), although this view is not widely accepted. And finally, anandamide, which blocks connexin channels previously suspected to be involved in taste signalling, does not interrupt communication within the taste bud (Huang et al. 2007).

CalHM1 is a non-selective pore-forming ion channel that can be activated either by lowering extracellular calcium, or in a voltage-dependent manner (Ma et al. 2012). There is evidence of CalHM1 expression in taste buds, as well as the

loss of CalHM1 reducing taste-evoked ATP release from type II taste cells in the tongue, which are normally sensitive to sweet, umami and bitter tastes (Taruno et al. 2013).

1.4.3. P2 receptors

The ATP signal from taste or glial cells is picked up by nearby cells expressing P2 receptors. The P2 purine receptor family is divided into two subfamilies: P2X, ligand-gated ion channels, and P2Y, which are GPCRs (Fredholm et al. 1994).

Structurally, P2X receptors are similar to other classical ligand-gated channels, and they form heteromeric complexes of structurally related subunits (Ralevic and Burnstock 1998). P2X receptors are found on various excitable cells – smooth muscle cells, neurons, and glia – and they act as mediators of excitatory neurotransmission in central and peripheral nervous systems (Saïag et al. 1990, Cotrina et al. 2000, Pankratov et al. 2003).

P2Y receptors are GPCRs (Webb et al. 1993). Five P2Y receptors have been fully characterised: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$. Human $P2Y_4$ and $P2Y_6$ receptors are uridine nucleotide-specific, only sensitive to UTP and UDP; rat $P2Y_4$ can also be activated by ATP (Communi et al. 1996, Communi et al. 1996, Soto et al. 1996). P2Y receptors exhibit a slower onset of response compared to P2X receptors (<10 ms vs. <100 ms) (Ralevic and Burnstock 1998).

1.5. Taste receptors in tanycytes

Tanycytes are glucosensitive, and they have been hypothesized to sense glucose by utilizing a mechanism similar to that of pancreatic β cells, consisting of specific glucose transporters, glucokinase and K_{ATP} channels, which are all present in tanycytes (García et al. 2003). Direct evidence from both cultured tanycytes and cells in acute brain slices shows that tanycyte glucosensing occurs via an ATP receptor-dependent mechanism (Frayling et al. 2011, Orellana et al. 2012) and, in a large proportion of tanycytes, also depends on the sweet taste receptor (Tas1r2/Tas1r3 heterodimer) (Benford et al. 2017). Tas1r2/Tas1r3 is normally found in the taste cells of the tongue, surrounded by

cells expressing receptors for other taste modalities (Nelson et al. 2001). A closely related receptor, the Tas1r1/Tas1r3 heterodimer, which shares a subunit with the sweet taste receptor, contributes to the perception of umami taste (savoury taste of glutamate in humans, or most non-aromatic L-amino acids in rodents) (Nelson et al. 2002). This has led to the hypothesis that hypothalamic tanycytes use umami taste receptors to detect amino acids in the cerebrospinal fluid; and changes in amino acid intake can alter tanycyte gene expression and responses to amino acids. As amino acids are important signals of satiety, determination of whether tanycytes might detect amino acids would be an important advance in understanding the energy homeostasis-related functions of these cells.

1.6. Experimental aims

The three main aims of this thesis are to:

1. Understand the complete signalling processes involved in tanycyte amino acid sensing, identify the receptors used and compare the findings to tanycyte sweet compound detection;
2. Determine the effects of different diets (fasting, chow, essential amino acid deficient) on tanycyte taste receptor expression and function;
3. Develop a link between tanycyte chemosensing, energy homeostasis and feeding behaviour in living animals.

2. Hypothalamic tanycytes sense amino acids via a Ca^{2+} and ATP-dependent pathway

2.1. Introduction

Hypothalamic tanycytes have recently emerged as chemosensitive, diet-responsive glial cells in the wall of the 3V, potentially involved in the regulation of energy homeostasis. They express receptors for peripheral signals associated with metabolism such as leptin and ghrelin (Balland et al. 2014, Collden et al. 2015), as well as the sweet taste receptor (Tas1r2/Tas1r3) to detect glucose (Benford et al. 2017). Considering that tanycytes contact the ventricular cerebrospinal fluid and are positioned outside of the blood-brain barrier, they could be able to detect not only glucose, but also the derivatives of the two other types of macronutrients – amino acids from protein, and fatty acids from fat. The fact that tanycytes use the sweet taste receptor suggests that there could be other similar receptors expressed in these cells.

The sweet taste receptor and the closely related umami taste receptor Tas1r1/Tas1r3 are GPCRs, which are characterised by the presence of seven membrane-spanning domains, an extracellular N terminus, and an intracellular C terminus (Rosenbaum et al. 2009). In these particular taste receptors, the binding of the tastant to the extracellular amino-terminal domain activates the heterotrimeric G proteins gustducins or $G\alpha_{i2}$, which stimulate PLC-β2. This in turn hydrolyses PIP2 to produce intracellular messengers IP_3 and diacylglycerol, which induce intracellular Ca^{2+} release, leading to the gating of the TRPM5. Mice lacking gustducin, PLC-β2, or TRPM5 have impaired sensitivity to sweet and umami taste, as well as bitterness (Chandrashekar et al. 2006).

The other known umami taste receptors mGluR1 and mGluR4 are also GPCRs but respond to amino acids by activating phosphodiesterases and decreasing intracellular cAMP, which disinhibits nucleotide monophosphate-inhibited channels to elevate intracellular Ca^{2+}. The effects of the Ca^{2+} increase are the same as for Tas1r1/Tas1r3, and eventually lead to ATP release.

ATP is most likely released from both taste receptor cells and tanycytes via large conductance membrane channels. Panx1 and CalHM1 have both been described as likely candidates for taste receptor cell ATP signalling (Dando and Roper 2009, Taruno et al. 2013). The ATP signal triggers a response in nearby taste nerve fibres (Yasumatsu et al. 2012), which then carry the information to the NTS (Whitehead and Frank 1983).

In my Master's thesis, I describe the initial experiments performed for this project that show tanycytes responding to a range of amino acids; the dependence of the response on both intracellular and extracellular Ca^{2+}; the involvement of a range of P2 receptors in the response pathway (discovered using P2X and P2Y receptor antagonists separately and as a mixture), particularly $P2Y_1$, $P2X_7$, and at least one other P2X receptor (likely $P2X_2$, as its expression can be seen very clearly in tanycytes throughout 3V in a previous study (Colldén et al. 2010)); and that the responses to Arg are blocked by CBX, a non-specific large conductance membrane channel inhibitor, known to block connexin hemichannels at high concentrations (≥100 μM) and pannexin 1 at low concentrations (≥10 μM). While these results pointed towards a GPCR-dependent pathway for tanycyte amino acid sensing, they also raised more questions, such as how sensitive tanycytes are to different amino acids, and how (and where) exactly ATP is released from these cells. This chapter aims to answer these questions.

The data presented in this chapter have been published as part of Lazutkaite *et al.*, 2017.

2.2. Methods

2.2.1. Brain slices

Male Sprague-Dawley rats (13–21 days old) were humanely killed in accordance with the UK Animals (Scientific Procedures) Act 1986. The brain was rapidly dissected and placed in ice-cold artificial cerebrospinal fluid (aCSF: 124 mM NaCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 3 mM KCl, 2 mM $CaCl_2$, 1 mM MgSO4, 10 mM glucose; saturated with 95% O_2/5% CO_2; osmolarity 300 mOsm) with additional 10 mM magnesium. 300 μm (for Ca^{2+} imaging) and 400

μm (for ATP biosensing) thick coronal sections were cut in the area of interest using a Microm HM 650 V vibrating blade microtome. Slices containing the hypothalamus were cut in half down the midline to the dorsal end of third ventricle leaving its walls intact, and through the median eminence to expose the tanycyte layer. These were then put into aCSF at 34–37˚C to incubate for 30–45 minutes and later transferred to a holding chamber with room temperature low-glucose (1 mM glucose + 9 mM sucrose to maintain osmolarity at 300 mOsm) aCSF and a constant supply of 95% O_2/5% CO_2, where they were maintained throughout the experiment.

2.2.2. Solutions and drug application

Amino acids and drugs were applied via either the bathing medium or series of 16 manual 300 ms puffs (pressure ~0.75 bar) from a patch pipette with a 2–3 μm tip, applied roughly every second for Ca^{2+} imaging, or a single 700 ms puff for ATP biosensing. This series of short puffs, as established in prior work (Frayling et al. 2011), superfuses the desired substance over the edge of the slice at a steady state concentration approximately 30–40-fold lower than the concentration within the pipette. The positioning of the brain slice and the pipette in the flow chamber is shown in Figure 2.1.

The amino acids used were L-alanine (Acros Organics, US), L-arginine (Acros Organics, US), L-lysine (Fisher Scientific, US), L-proline (Merck, Germany), and L-serine (Sigma-Aldrich, UK). The concentration of the amino acids in the puff pipette was 150–300 mM, depending on osmolarity, which was maintained around 300 mOsm to match aCSF. All amino acid solutions were made in purified H_2O (R = 18.2 MΩ). 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Sigma-Aldrich, UK) buffer was added to 165 mM L-arginine to maintain neutral pH. According to previous experiments using glucose biosensors placed at the edge of the brain slice during puffs of 300 mM glucose, the final concentration of glucose reaching the slice was 30–40 times lower than the concentration in the pipette depending on the distance from the tip of the pipette to the tanycytes (Frayling et al. 2011). As we were unable to obtain sensors for each amino acid, we used the glucose data to calculate approximate concentrations of amino acids, and they will be referred

to further in the text as: 5 mM and 10 mM L-alanine, 5 mM L-arginine, 5 mM L-lysine, 5 mM and 10 mM L-proline, 5 mM and 10 mM L-serine.

The concentrations of the drugs used were as follows: 200 μM 10panx (Tocris Bioscience, UK), 10 μM BBG (Sigma-Aldrich, UK), 10 μM and 100 μM CBX (Sigma-Aldrich, UK), 165 μM GAP 26 (VWR International Ltd., UK), 100 nM MRS2500 (Tocris Bioscience, UK), 50 μM Suramin (Sigma-Aldrich, UK), 30 μM PPADS (Tocris Bioscience, UK), 1 mM Probenecid (Sigma-Aldrich, UK), 50 μM Ruthenium Red (Sigma-Aldrich, UK), 1 μM tetrodotoxin (TTX; Tocris Bioscience, UK). The solutions were prepared in aCSF except for Probenecid, which had to be dissolved in 1 mM NaOH and then neutralised with HCl prior to adding it to aCSF. Only one drug treatment was performed per slice to avoid loss of responses due to overstimulation, bleaching of Fura-2, or loss of cell viability.

Bath application of all substances except 10panx and Gap26 included incubation in the flow chamber for 10–30 minutes without imaging. When using 10panx and Gap26, each slice was tested for a control response to 5 mM L-arginine, then taken out of the flow chamber and put into an incubation chamber with either regular low-glucose aCSF (control) or low-glucose aCSF with 10panx or Gap26 for 30 minutes, and put back into the flow chamber, keeping the orientation of the slice in the chamber and the position relative to the puff pipette as similar to the initial set up as possible. Responses to L-arginine were recorded within 5 minutes from the end of incubation to prevent washout of the peptides.

2.2.3. Imaging

Prior to imaging, the slices were incubated for 75-95 minutes in 1.25 μM Fura-2 AM (Fura-2 dissolved in 20% Pluronic F-127 solution in DMSO and diluted 1:800 with low-glucose aCSF; Life Technologies, UK). After incubation, the slices were transferred into a holding chamber with low-glucose aCSF to wash out excess Fura-2 AM and DMSO/Pluronic. For imaging, they were transferred to a flow chamber on an Olympus BX51 microscope equipped with a 60x water immersion objective (NA 1.0). Ratiometric imaging of Fura-2 emissions at

excitation wavelengths of 340 and 380 nm (provided by a Cairn Research Optoscan monochromator) was performed under the control of the MetaFluor software. Example images where 6 individual cells can be identified and corresponding region of interest (ROI) traces from these cells are shown in Figure 2.1 B,C.

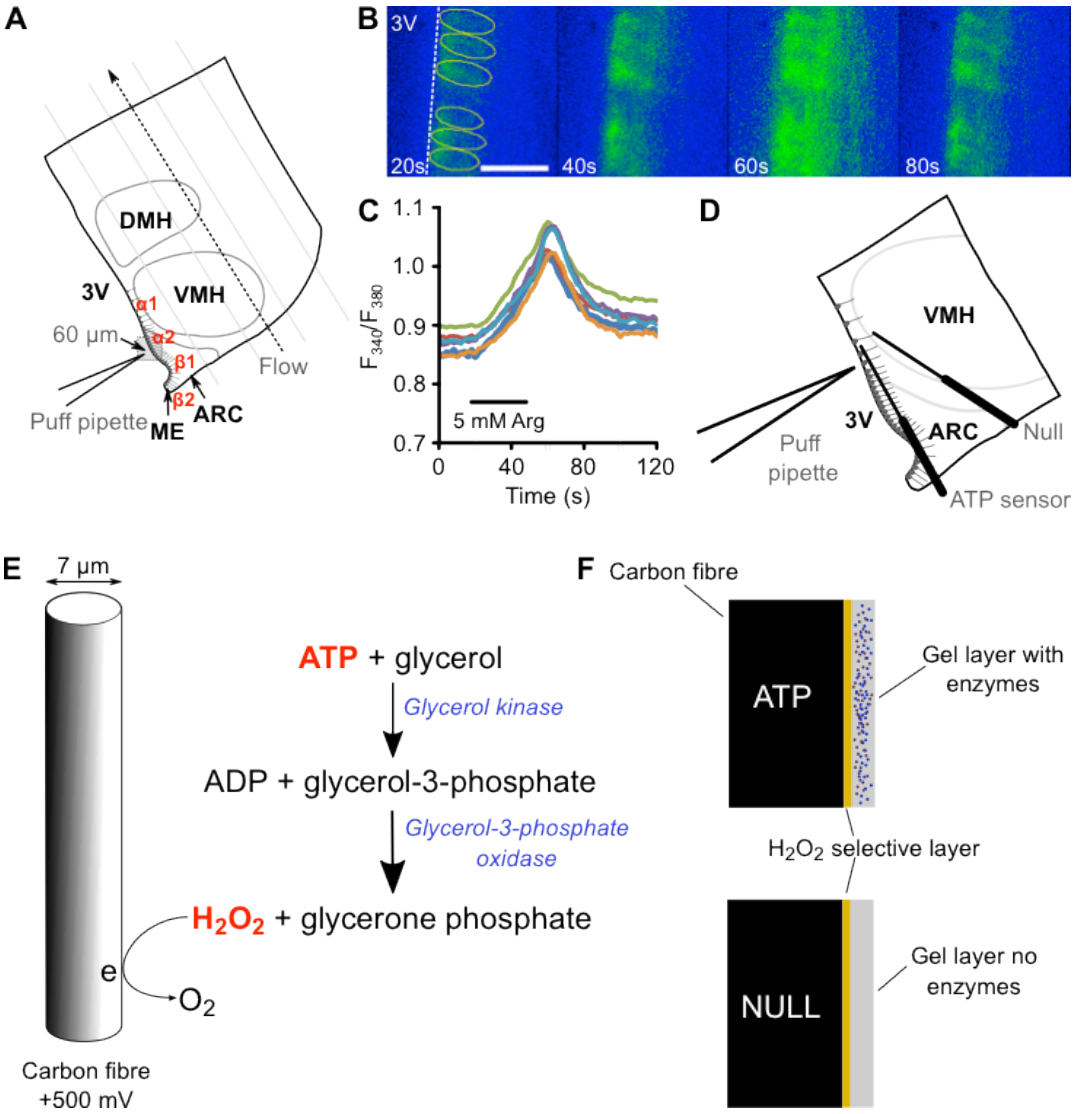

Figure 2.1. Set up for Ca^{2+} imaging and biosensing. *(**A**) Schematic representation of the arrangement of a brain slice and puffing pipette in the imaging chamber. (**B**, **C**) Individual tanycytes can be seen responding to Arg under higher magnification. ROIs in the graph represent the cells seen in the montage. Scale bar 20 µm. (**D**) Schematic representation of the arrangement of a brain slice, puffing pipette and microelectrode biosensors in the flow chamber. (**E**) Enzymatic scheme of the ATP biosensor. The cascade of enzyme reactions produces H_2O_2 when ATP is present. Glycerol is added to the bathing medium prior to ATP measurements. H_2O_2 is oxidized on the electrode surface (polarized to 500 mV). (**F**) The Null sensor is an exact copy of the ATP biosensor without the enzymes required for ATP detection. It is therefore only sensitive to electrical disturbances and non-specific events, allowing exclusion of any noise or artefacts from the ATP biosensor recording. For more information see Llaudet et al., 2005. DMH, dorsomedial hypothalamic nucleus; 3V, third ventricle; VMH, ventromedial hypothalamic nucleus; ARC, arcuate nucleus; ME, median eminence.*

2.2.4. ATP biosensing

For direct biosensing of ATP in brain slices, custom-made enzyme-coated 7 µm carbon fibre microelectrodes (Sarissa Biomedical LTD, UK) were inserted into the hypothalamic tissue immediately next to tanycyte cell bodies, contacting the bases of tanycyte processes. A “null” electrode (exact copy of the ATP biosensor lacking the enzyme layer, only sensitive to non-specific events and noise – see Figure 2.1 F) was placed next to the biosensor and used as a reference for any mechanical or electrical disturbances during the recordings. The final values of the measurements were calculated by subtracting the “null” values from the biosensor values at each time point. The arrangement of the electrodes in the brain slice is shown in Figure 2.1 D.

The biosensor and the “null” were connected to a Sycopel Duo-Stat ME 200+ potentiostat. The biosensor traces were recorded via a Data Translation DT3016 AD board and custom software was used for storage and analysis.

2 mM glycerol was added to all the bathing solutions used in these experiments to allow the enzymes within the biosensor – glycerol kinase and glycerol-3-phosphate oxidase – produce hydrogen peroxide when ATP is present (Fig 2.1 E,F) (Llaudet et al. 2005).

The biosensors were calibrated with a 50 µM ATP solution in aCSF at the start and the end of each day, as well as in the middle of the experiments or whenever a new biosensor was used.

2.2.5. Data analysis

The emission ratios for F_{340}/F_{380} were calculated using ImageJ software for every individual tanycyte visible in the field (selected manually). The baseline was calculated from 15 images prior to the start of the amino acid application and maximum change in the intensity in each region of interest was found for every trial (when substances potentially altering tanycyte responses were used, trials were described as follows: CONTROL amino acid application; DRUG – amino acid application in the presence of another substance; and WASH – amino acid application after washing the slice in regular aCSF for an extended

period of time). Average responses were then calculated for each trial in each slice.

The data from the biosensor recordings were analysed using proprietary software. The amplitudes of the response peaks were measured manually and then compared to the amplitude of the biosensor calibration curve recorded using a known amount of ATP (50 μM) to calculate the highest amount of ATP detected during each response.

Graphpad Prism 7 was used to produce graphs. All values represented in the graphs in this and following chapters can be found in the appendix.

2.2.6. Statistical analysis

Statistical analysis was performed using Graphpad Prism 7 software. For experiments investigating the effects of drugs, a single brain slice was considered an independent replicate (the total number of animals that the slices were obtained from is given in brackets). The Friedman non-parametric 2-way ANOVA was used to compare the responses across the CONTROL, DRUG and WASH trials. Median responses in both Ca^{2+} imaging and ATP biosensing experiments were compared between the CONTROL and DRUG trials using Wilcoxon's matched pair signed ranks test. The WASH trial was used to evaluate the loss of cell vitality and responsiveness due to the length of the experiment and the number of amino acid applications. Exact probabilities calculated by the tests are reported in text or figures.

All statistical tests were two-sided. Data collection and analysis were not performed blind to the conditions of the experiments.

2.3. Results

2.3.1. Tanycytes respond to L-amino acids with a Ca^{2+} wave

Fura-2 AM-loaded hypothalamic tanycytes displayed an increase in intracellular calcium in response to a series of puffs of L-amino acids. The highest responses to 5 mM solutions of L-amino acids were observed when using essential amino acids Lys and Arg (Figure 2.2, 2.3). No response was observed when Arg was puffed directly onto the brain parenchyma, i.e. ARC or VMH,

instead of the tanycyte layer (mean ± SEM amplitude $\Delta F_{340}/F_{380}$ 0.032 ± 0.020, n = 5 slices from 4 animals; example ROI trace shown in Figure 2.3 C). Non-essential amino acids Ala, Pro and Ser only elicited minor responses at 5 mM (Figure 2.4 B, 2.5 B, 2.6 B, left panels). As shown in Figures 2.4–2.6, increasing the concentration of non-essential amino acids to 10 mM resulted in higher responses to Ala and Ser. 10 mM Ala responses reached similar overall amplitude to the essential amino acids, but with higher variability (Figure 2.6 C). The response to 10 mM Pro, which is a weak agonist for the suspected tanycyte amino acid receptors Tas1r1/Tas1r3, mGluR1 and mGluR4, was still lower than the responses to all other tested amino acids (Figure 2.6 B,C).

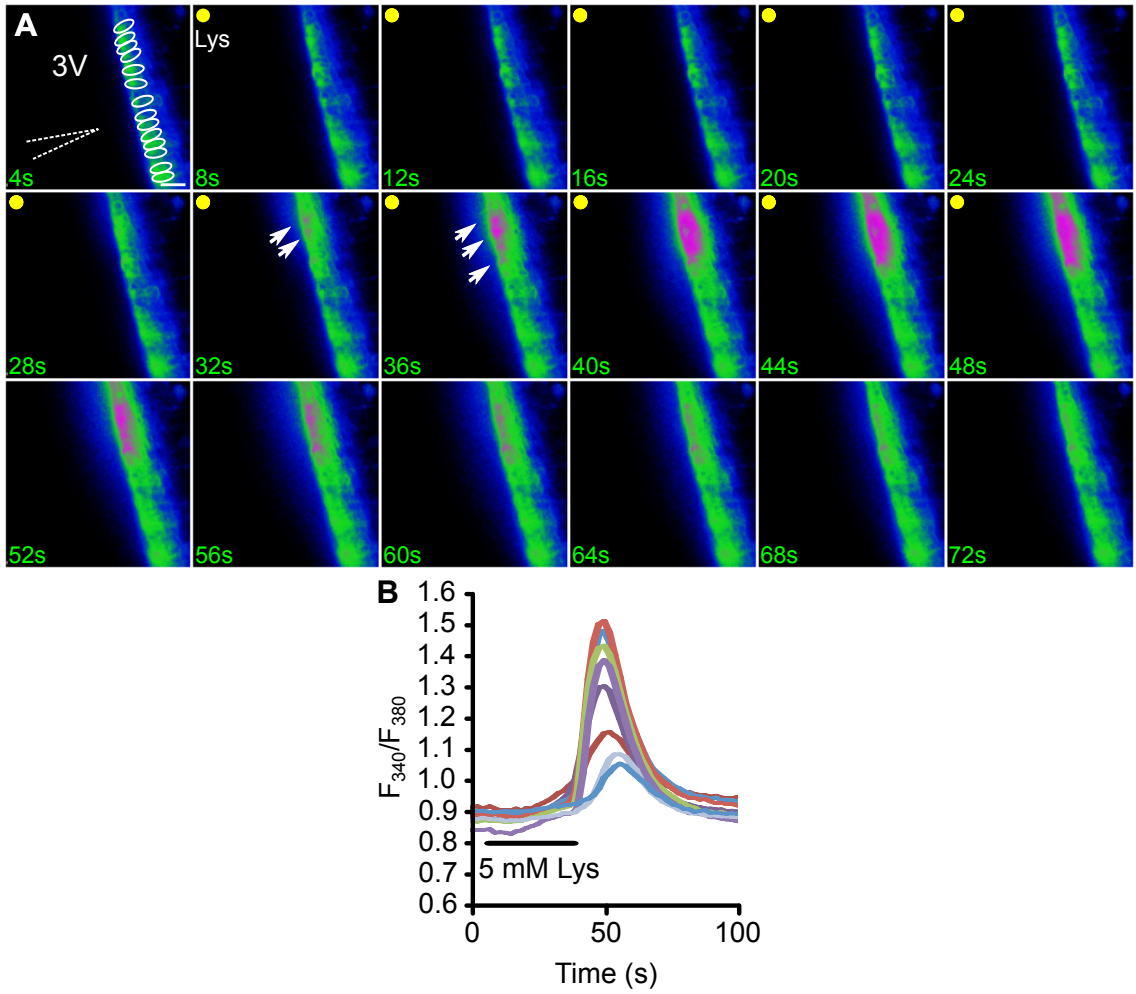

Figure 2.2. Tanycytes respond to L-lysine. *(**A**) Montage and (**B**) corresponding ROI trace show an example response to 5 mM Lys. Yellow dots on the montage and black lines on the trace indicate puffing duration. White arrows show the spread of the Ca^{2+} wave. Scale bar 20 µm.*

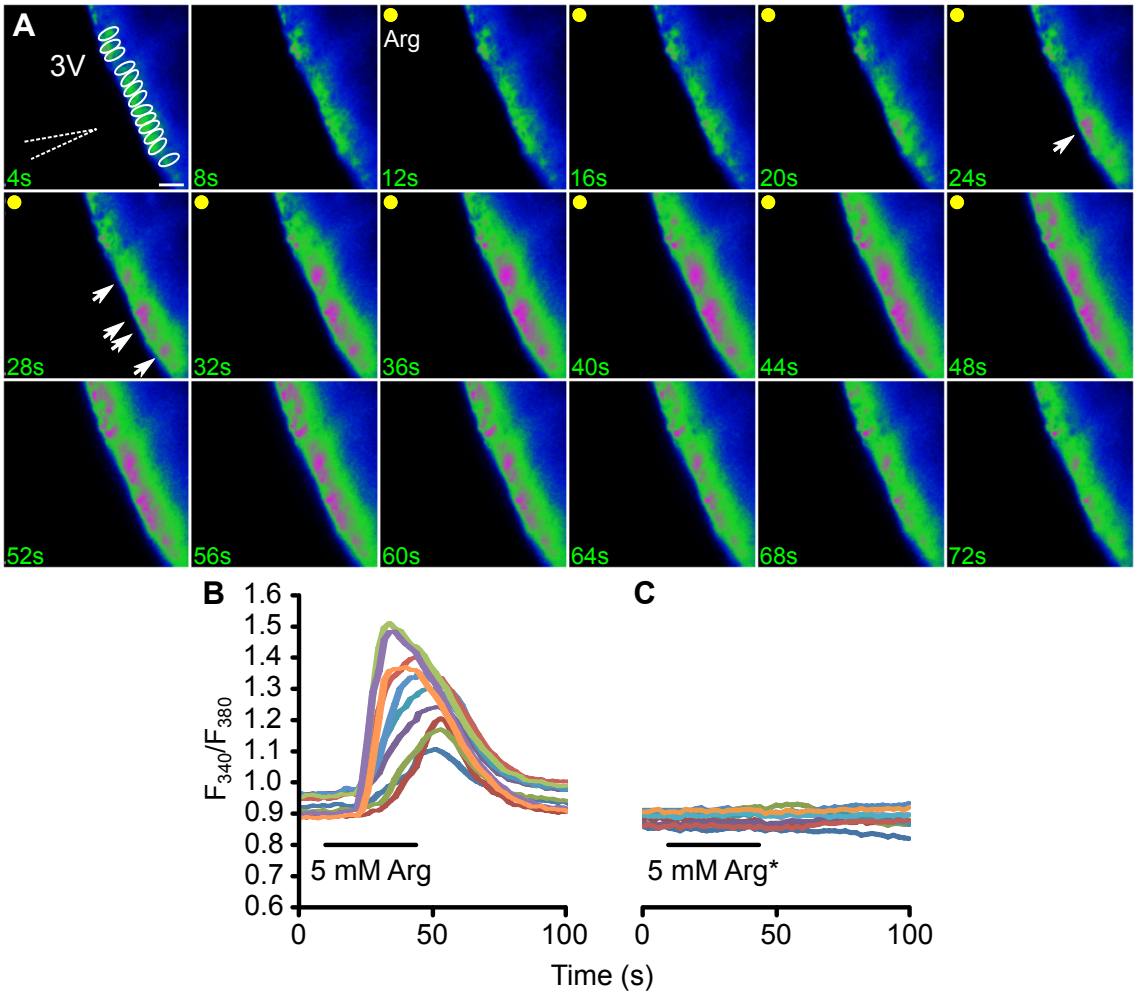

Figure 2.3. Tanycytes respond to L-arginine. *(**A**) Montage and (**B**) corresponding ROI trace show an example response to 5 mM Arg. Yellow dots on the montage and black lines on the trace indicate puffing duration. Scale bar 20 µm. (**C**) 5 mM Arg does not elicit a tanycyte response when applied directly on the brain parenchyma.*

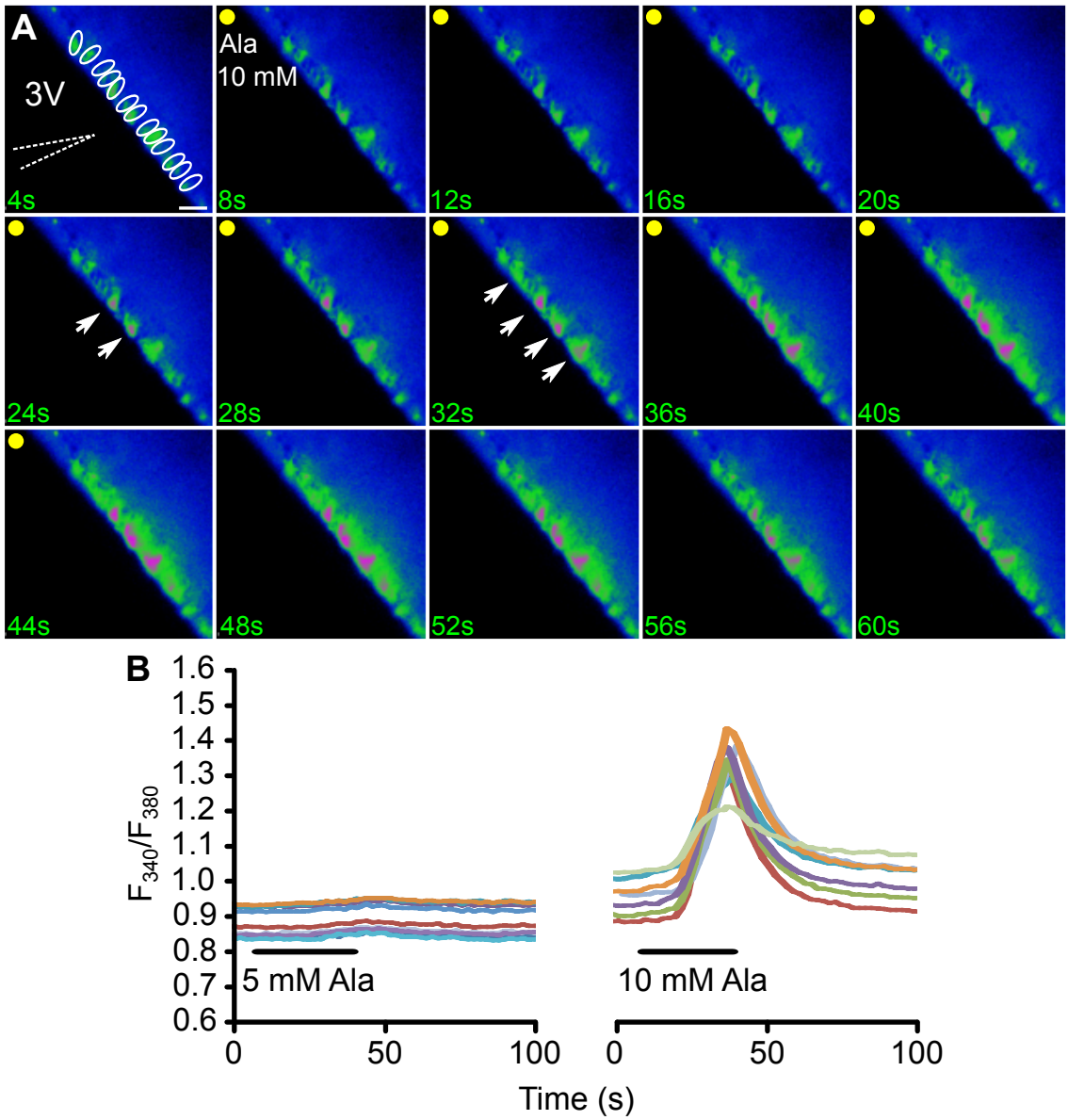

Figure 2.4. Tanycytes respond to L-alanine. *(**A**) Montage showing example responses to 10 mM Ala. (**B**) ROI traces showing example responses to 5 mM and 10 mM Ala. Yellow dots on the montages and black lines on the traces indicate puffing duration. Scale bar 20 µm.*

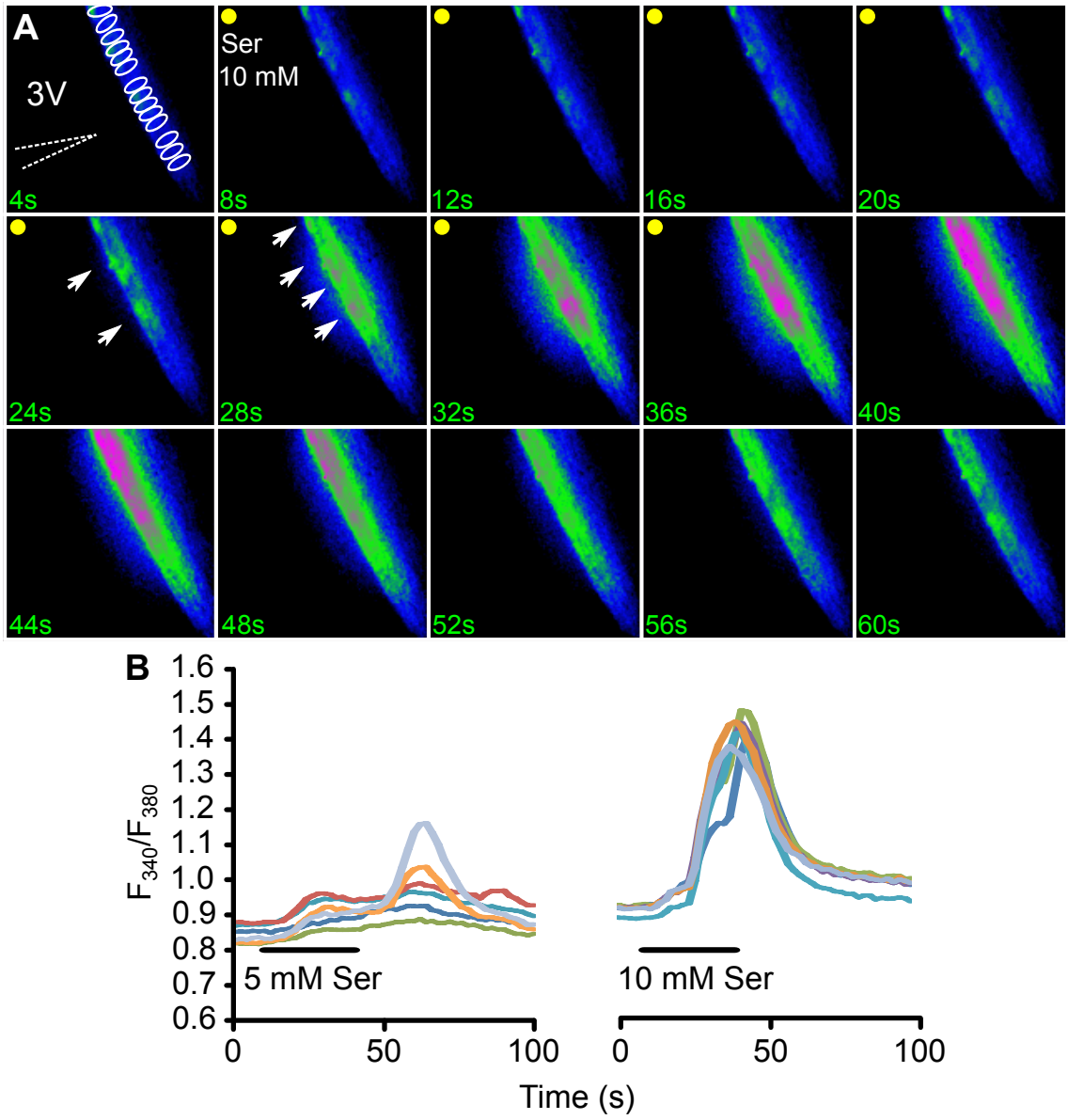

Figure 2.5. Tanycytes respond to L-serine. *(**A**) Montage showing example responses to 10 mM Ser. (**B**) ROI traces showing example responses to 5 mM and 10 mM Ser. Yellow dots on the montages and black lines on the traces indicate puffing duration. Scale bar 20 µm.*

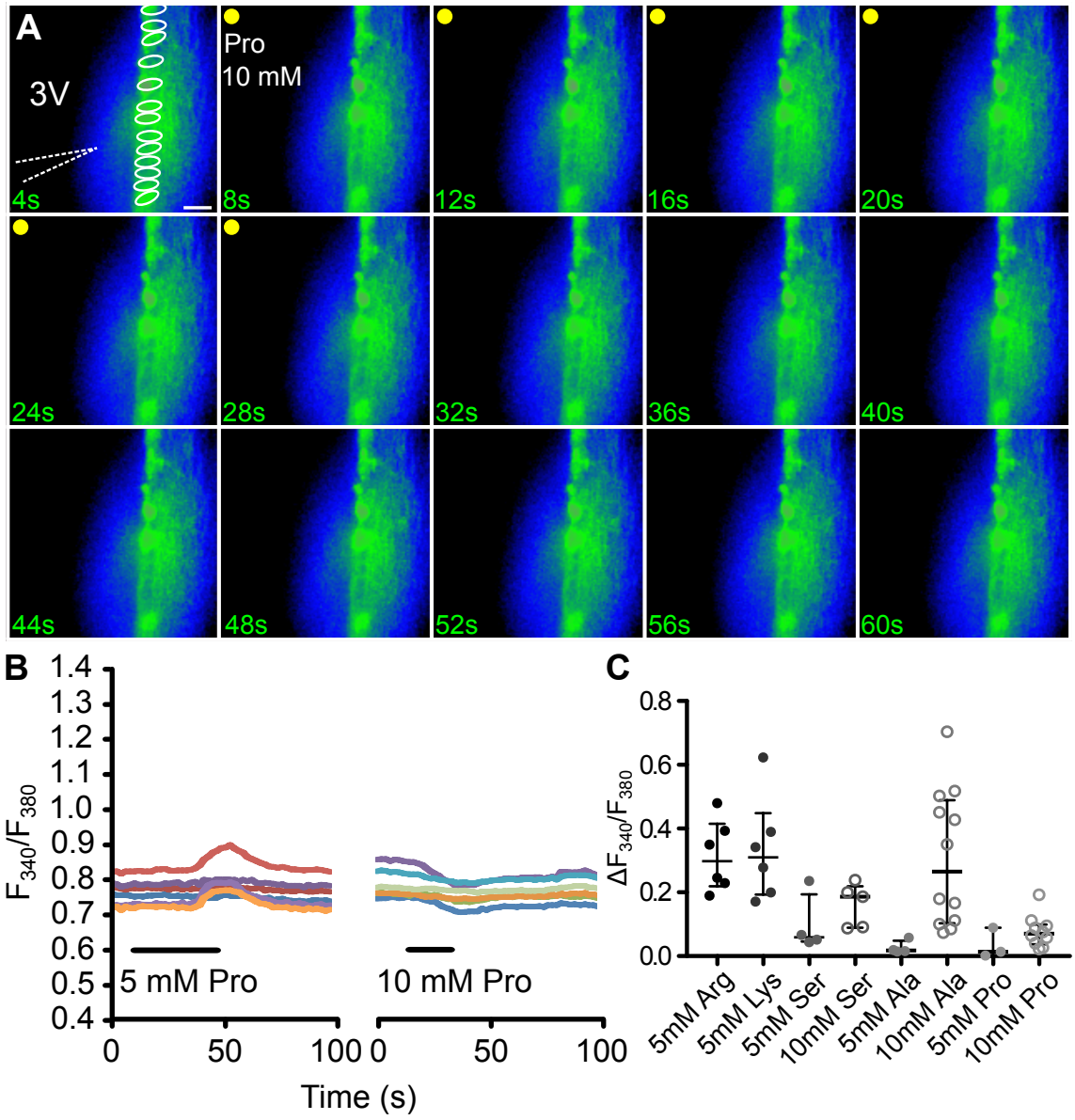

Figure 2.6. Tanycytes have low sensitivity to L-proline. *(**A**) Montage showing example responses to 10 mM Ser. (**B**) ROI traces showing example responses to 5 mM and 10 mM Ser. Yellow dots on the montages and black lines on the traces indicate puffing duration. Scale bar 20 µm. (**C**) Full tanycyte amino acid response profile.*

To further confirm that the Ca^{2+} wave originated from tanycytes, the Na^{+} channel inhibitor TTX (Narahashi 1974) was used to prevent any potential signals generated by nearby neurons. TTX did not block tanycyte responses to Arg (Figure 2.7), confirming that tanycyte sensitivity to amino acids does not depend on spiking in neurons.

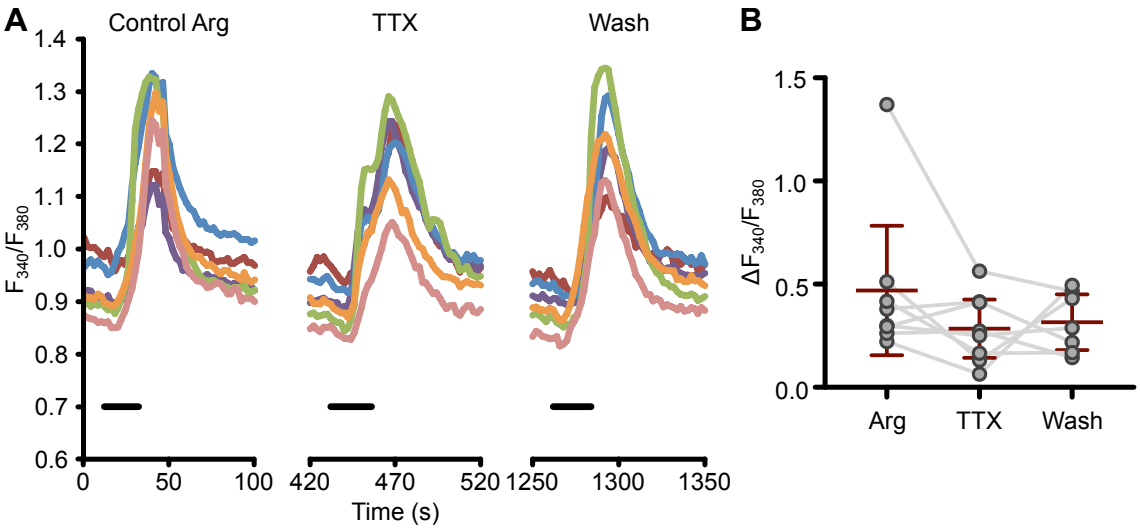

Figure 2.7. Tanycyte responses to L-amino acids are independent of neuronal activity. *(**A**) Example ROI traces from 1 slice and (**B**) graph showing that tetrodotoxin (TTX), a Na^+ channel inhibitor, did not reduce tanycyte responses to Arg.*

2.3.2. Tanycytes release ATP in response to L-amino acids

The responses to amino acids in taste receptor cells of the tongue follow an ATP receptor-dependent pathway (Finger et al. 2005). In the work conducted during my MSc, a range of P2 receptor antagonists were used to prevent the spread of the tanycyte signal along the edge of 3V. Based on the findings of Frayling *et al*. and Benford *et al*., who showed $P2Y_1$ expression and involvement in sweet taste signalling in tanycytes, the $P2Y_1$ antagonist MRS2500 was first used to reduce tanycyte responses to amino acids, but had no effect (Figure 2.8 A,B). PPADS, Suramin, and Brilliant Blue G (BBG), which act on several P2X and P2Y receptors, were then tested but individually, none of these antagonists blocked tanycyte signals (Figure 2.8 C). However, combinations of any tested P2X antagonist and MRS2500 successfully reduced tanycyte responses to Arg (Figure 2.8 D–F; time-matched control shown for comparison in Figure 2.8 E). This suggested that multiple ATP receptors, one of which was $P2Y_1$, were required to elicit a Ca^{2+} wave in response to Arg. The lack of effect from individual antagonists, therefore, may be explained by the compensation by other P2 receptors involved in mediating tanycyte amino acid responses. These findings were used as the basis for further experiments on tanycyte ATP signalling.

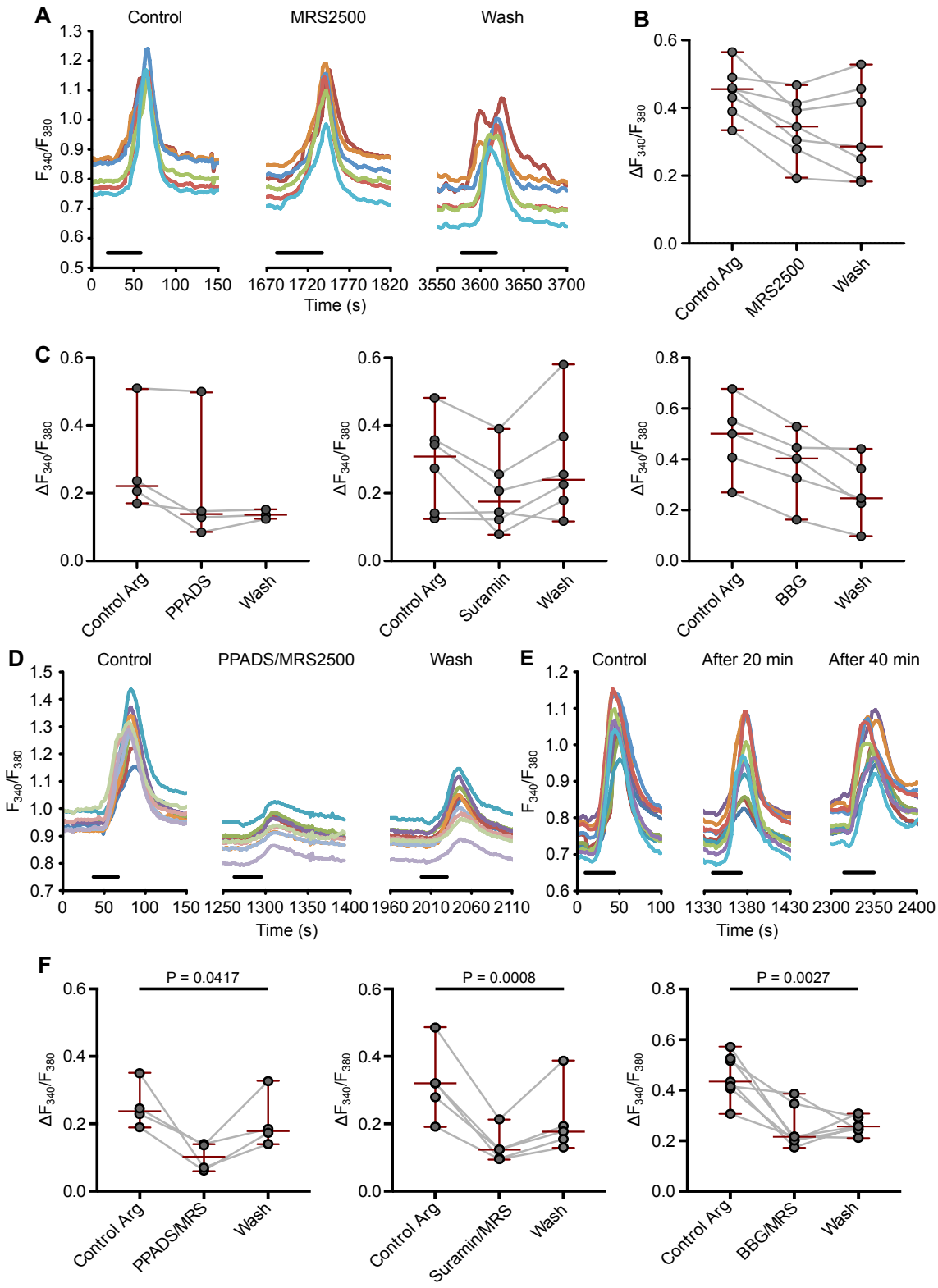

Figure 2.8. Tanycyte responses to L-amino acids require ATP detection via P2 receptors. *(**A, B**) Inhibition of P2Y$_1$ with MRS2500 was not sufficient to block tanycyte responses to Arg. (**C**) Non-specific P2 receptor antagonists PPADS, suramin and BBG failed to inhibit tanycyte responses. (**D–F**) Combinations of P2X and P2Y antagonists reversibly blocked tanycyte responses to Arg. (**E**) Time-matched control shows that the blockage was specific to the drugs and not dependent on the time intervals between amino acid applications. P values given for Friedman test comparing all three trials.*

Direct biosensing using custom made 7 µm carbon fibre biosensors (200 µm in length; Sarissa Biomedical Ltd, UK) showed an increase in ATP next to tanycyte cell bodies immediately after a single amino acid puff. A single 700 millisecond puff was enough to elicit ATP release in response to Ala, Arg and Ser (example Ala and Arg traces in Figure 2.9 B). Series of puffs resulted in saturation of the response, as the cells could only produce a limited amount of ATP in a short period of time (data not shown). Responses were observed only when amino acids were applied directly on the tanycyte layer, but not on brain parenchyma (Figure 2.9 C,D).

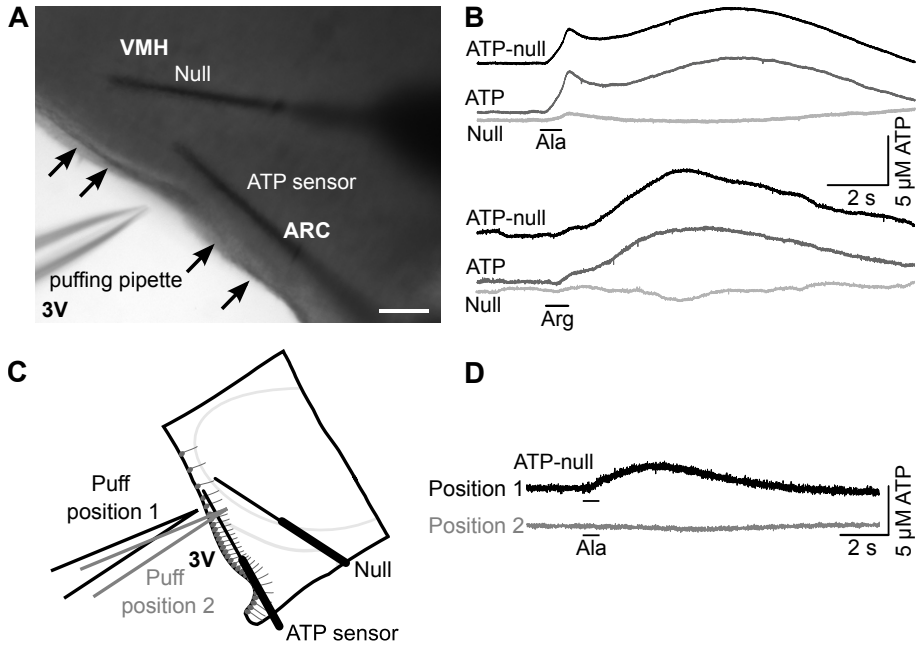

Figure 2.9. ***Tanycytes release ATP in response to L-amino acids.*** *(**A**) The arrangement for the biosensor recordings. Black arrows indicate the tanycyte layer. Scale bar 100 µm. VMH, ventromedial hypothalamic nucleus; ARC, arcuate nucleus; 3V, third ventricle. (**B**) Biosensor and "null" traces and the corrected response curve ("ATP-null") from tanycytes responding to 10 mM Ala and 5 mM Arg. (**C**) Schematic representation of the biosensor and puff pipette positions for applying amino acids directly on tanycyte bodies (position 1) or on the parenchyma (position 2) (**D**) A comparison of ATP release detected when 10 mM Ala was applied directly on tanycyte bodies (position 1) and on the parenchyma (position 2). VMH, ventromedial hypothalamic nucleus; ARC, arcuate nucleus; 3V, third ventricle.*

The increase in ATP concentration (estimated against a calibration curve recorded with 50 µM ATP) after a single puff ranged between 0.3–10 µM. Any values higher than 10 µM were disregarded as potential artefacts, which could have occurred due to damage to the gel layer of the biosensor.

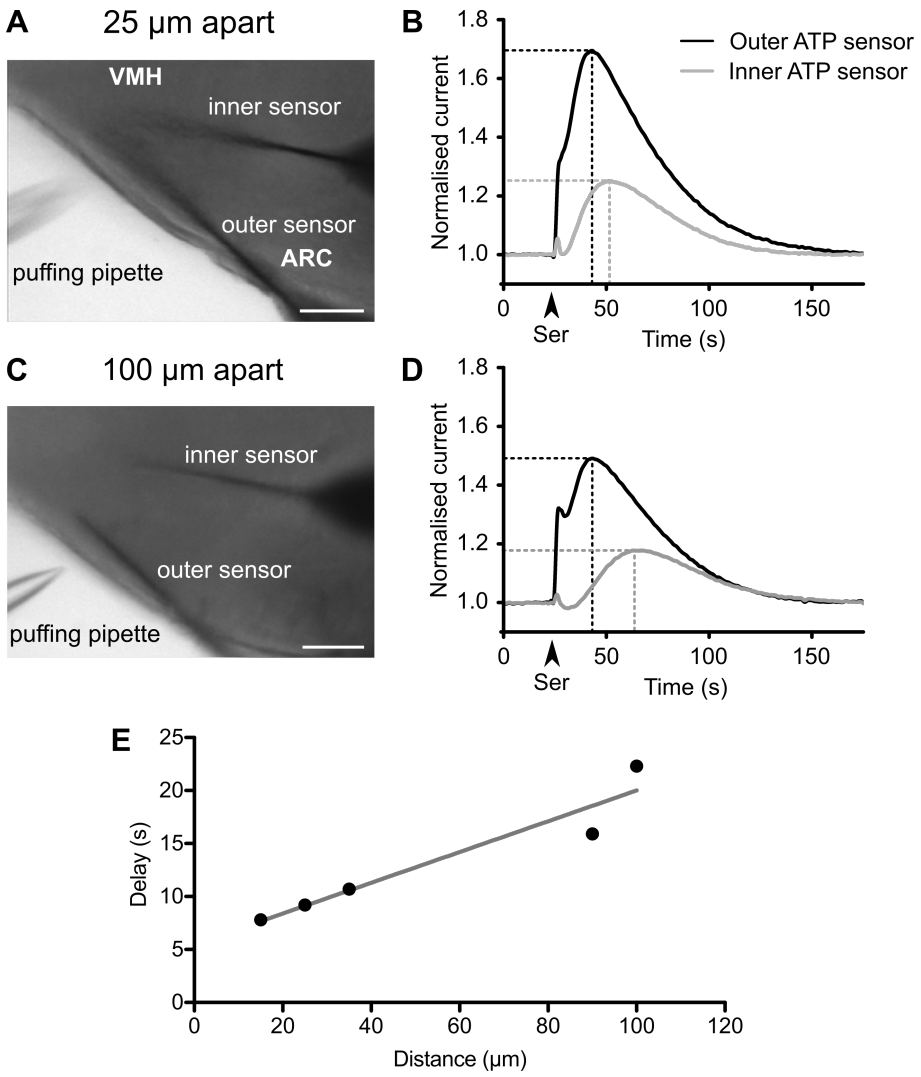

Figure 2.10. ATP travels down tanycyte processes. *(**A, C**) The arrangement for the biosensor recordings with a second (inner) biosensor placed at different distances relative to the first (outer) biosensor. Scale bar 100 µm. VMH, ventromedial hypothalamic nucleus; ARC, arcuate nucleus; 3V, third ventricle. (**B, D**) The amount of ATP detected by the two biosensors and the delay between the outer and inner biosensor response peaks depended on the distance between the biosensors (**E**) ATP travelled into the brain parenchyma at a consistent rate.*

To trace the movement of the ATP signal, combined measurements with 2 biosensors (a second biosensor in place of “null”) were performed. The second sensor was placed at a varying distance from the tanycyte cell bodies (Figure 2.10). These measurements showed that the amount of ATP detected decreased moving the second sensor further into the hypothalamus, while the delay of the response peak increased. The wave of ATP travelled into the parenchyma at approximately 3.6 µm/s (SD = 1.48, n = 5; Figure 2.10 E). The speed of the ATP wave is comparable to the speed at which Ca^{2+} moves down the tanycyte processes (Benford et al. 2017). This suggests that in response to

amino acids, tanycytes send an ATP signal down their processes towards the hypothalamic nuclei that control energy homeostasis.

2.3.3. ATP is released via large conductance channels

To further investigate the pathway of ATP release from hypothalamic tanycytes, mimetic peptides for two large conductance membrane channels – Panx1 and Cx43, both likely candidates for tanycyte taste signal transduction, – were used in an attempt to block the responses to amino acids. Gap26, a mimetic peptide for Cx43 (Desplantez et al. 2012), did not consistently reduce tanycyte responses to Arg, although a trend for reduction ($p = 0.065$ Wilcoxon's matched pair signed ranks test) could be seen (Figure 2.11 A). Blocking Panx1 with the mimetic peptide 10panx, on the other hand, resulted in a strong reduction in the responses to Arg, revealing a role for Panx1 in tanycyte amino acid detection (Figure 2.11 A,B).

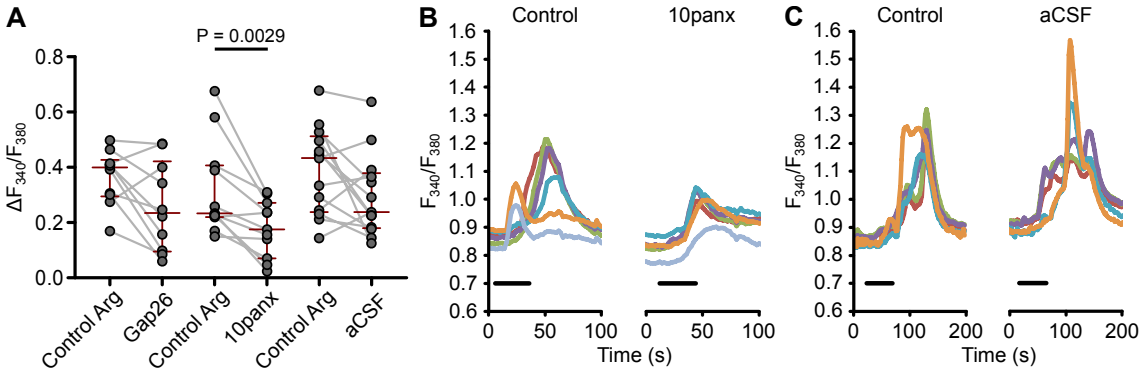

Figure 2.11. ATP is most likely released from tanycytes via pannexin 1 after L-arginine application. *(**A**) Gap26, the mimetic peptide for connexin 43, did not consistently reduce tanycyte responses to L-arginine. The pannexin 1 mimetic peptide 10panx weakened the responses (Wilcoxon signed ranks test). (**B**) Example ROI traces from tanycytes responding to L-arginine before and after incubation in 10panx and (**C**) a time-matched control. Black lines indicate puffing duration.*

Another channel, CalHM1, is also a potential conduit for tanycyte ATP release. Ruthenium red (RuR), Gd^{3+} and Zn^{2+} are known inhibitors of CalHM1 (Ma et al. 2012). RuR did not have an effect on tanycyte responses to Arg (Figure 2.12 A). However, RuR was also tested against Ala in the ATP biosensing experiments, this time reducing the amount of ATP released after an Ala puff (Figure 2.12 B). The results were then replicated with Ca^{2+} imaging (Figure 2.12 C,D), showing that CalHM1 is used for tanycyte ATP release in response to Ala, but not Arg.

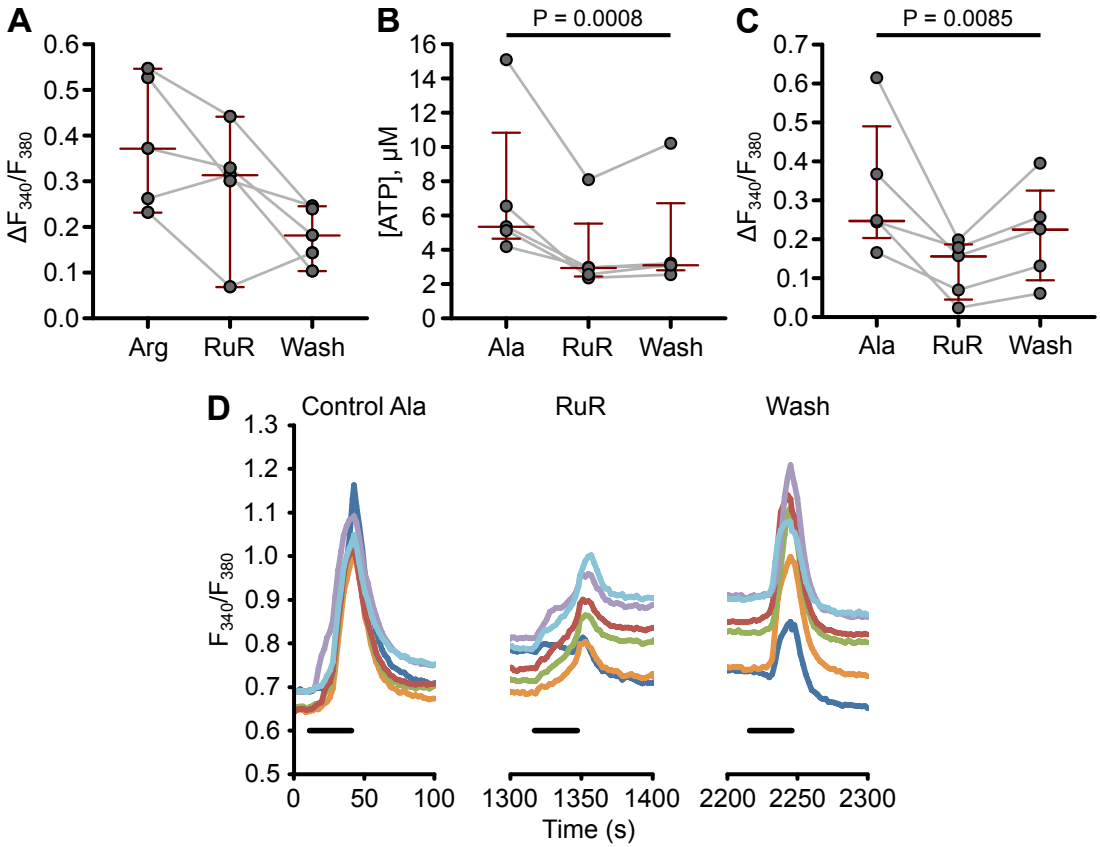

Figure 2.12. Tanycyte ATP release in response to L-alanine requires CalHM1. *(**A**) Ruthenium red (RuR), which blocks CalHM1, did not affect tanycyte responses to L-arginine. (**B**) Ruthenium red reduced biosensor-detected tanycyte ATP release in response to L-alanine. (**E, F**) The reduction in the response was also observed with Ca^{2+} imaging, confirming that tanycyte responses to L-alanine lead to ATP release trough CalHM1. P values given for Friedman test.*

2.4. Discussion

Rat hypothalamic tanycytes responded to a range of amino acids following the expected GPCR pathway and releasing Ca^{2+} and ATP, with involvement of P2 receptors and large, non-selective calcium channels.

Based on umami taste receptor sensitivity levels for different amino acids described in cultured taste receptor cells (Nelson et al. 2002), the expected outcome of the first experiment was that tanycytes would exhibit greater responses to non-essential (Ala, Ser) than essential (Arg, Lys) amino acids. The results contradicted this hypothesis as the concentrations of non-essential amino acids had to be increased 2-fold to induce responses that resembled those to Arg and Lys. This difference between amino acid sensing in the tongue and in tanycytes could occur due to minor structural differences in the taste receptors expressed in the different areas; for example, it is known that taste-

and brain- variants of mGluR4 have different glutamate binding sites as taste-mGluR4 has a truncated N-terminal, which results in lower affinity (Chaudhari et al. 1996). Similarly, the ligand binding sites of tanycyte Tas1r1/Tas1r3 could have evolved to detect essential amino acids with higher affinity in order to inform the hypothalamus about their availability.

2.4.1. Tanycytes exhibit specific responses to different L-amino acids

Distinct response patterns were observed with each tested amino acid. Arg and Lys elicited high responses at a low concentration (5 mM). Ala only worked at a higher concentration (10 mM), and the response was blocked by RuR, suggesting ATP release via CalHM1. Conversely, tanycyte responses to Arg were not reduced by RuR and seemed to depend on Panx1 with potential partial involvement of Cx43. Ser elicited slightly lower amplitude, slower responses than Arg or Lys observed in both Ca^{2+} imaging and ATP biosensing. The concentrations of Pro used in this experiment were not sufficient to activate tanycytes, suggesting that Pro is a weak agonist for tanycyte amino acid receptors.

The range of shapes and sizes of the Ca^{2+} and ATP waves, the previously demonstrated need for multiple P2 receptors, and the involvement of Panx1 and CalHM1 in the responses to different amino acids indicate that there must be more than one pathway for tanycyte amino acid detection. This could either be two or more separate receptors (similarly to Tas1r1/Tas1r3, mGluR1 and mGluR4 functioning together in the tongue), or agonist-dependent coupling from one receptor to different membrane channels.

In tanycyte sweet taste sensing, inactivating $P2Y_1$ with MRS2500 is sufficient to reduce tanycyte responses to glucose and artificial sweeteners mediated by Tas1r2/Tas1r3 (Benford et al. 2017). Tanycyte amino acid detection, on the other hand, involves more P2 receptors: a mix of antagonists that block a number of P2X receptors including $P2X_2$ and $P2X_3$, as well as $P2Y_1$, is required to eliminate tanycyte responses to Arg (Lazutkaite et al. 2017), which could be an adaptation to increase sensitivity as the relative levels of amino acids in the third ventricle are much lower than they are in the oral cavity during a meal. Moreover, as P2 receptors differ in their sensitivity for ATP and other

nucleotides, simultaneous activation of certain subsets of P2 receptors after the detection of different amino acids could relay substrate-specific information to ARC neurons.

Both Panx1 and CalHM1 have been shown to be involved in amino acid sensing in taste receptor cells of the tongue (Dando and Roper 2009, Taruno et al. 2013); however, there has not yet been a suggestion that they could be parts of separate detection mechanisms for different amino acids. Since none of the tested membrane channel antagonists fully abolished tanycyte responses to either amino acid, some overlap can be suspected between the two tanycyte amino acid signalling pathways.

2.4.2. Tanycyte ATP release could be used for tanycyte-to-neuron signalling

The ATP detection experiments demonstrated that ATP released by tanycyte cell bodies spreads not only to neighbouring cells, but also down the tanycyte processes and into the hypothalamic nuclei controlling food intake and metabolism. This is an important finding as it suggests potential nutrient-dependent tanycyte-to-neuron signalling, which has not been described before. Experimental evidence from mice shows that optogenetic stimulation of tanycytes expressing Ca^{2+} permeable channelrhodopsin (CaTCh) depolarizes nearby NPY and POMC neurons and increases the frequency of spontaneous depolarizing post-synaptic potentials depending on the metabolic state of the subject (Dr. M. Bolborea, personal communication, September 2017). While the optogenetic signal is different in many aspects from the amino acid signal, the ATP released from tanycytes in response to dietary amino acids could potentially be used to inform these neurons about nutrient availability in the 3V. Alternatively, tanycytes could be signalling to astrocytes (or other glial cells in the area), which would then influence neuronal activity accordingly.

The tanycyte signals observed in this study clearly indicate a role for tanycytes in central amino acid sensing, and they may possibly be responsible for the delivery of nutrient-related information to the neural circuits that regulate energy homeostasis. It could be speculated at this point that the tanycyte amino acid

signalling relates to satiety, as amino acids are a powerful indicator of food intake and, when administered centrally, can act on the hypothalamus to reduce hunger (Panksepp and Booth 1971).

3. The identification of amino acid receptors in tanycytes

3.1. Introduction

Hypothalamic tanycytes respond to L-amino acids via a GPCR-dependent pathway. It is now known that tanycytes express the sweet taste receptor Tas1r2/Tas1r3, which is a GPCR, to detect glucose and artificial sweeteners (Benford et al. 2017). A closely related umami taste receptor Tas1r1/Tas1r3 shares a subunit with the sweet taste receptor and is used in taste buds of the tongue to detect amino acids (L-glutamate in humans and a wide range of non-aromatic L-amino acids in rodents) (Nelson et al. 2002). However, Tas1r1/Tas1r3 is not the only amino acid receptor in the tongue, as research using mice lacking either subunit of the receptor or its downstream target TRPM5 showed that these animals were still able to taste amino acids, although the sensitivity was reduced; mGluR1 and mGluR4 have been suggested as additional umami taste receptors since blocking these receptors reduced the sensitivity of taste receptor cells to amino acids even further (Yasumatsu et al. 2012). The different umami taste receptors on the mouse tongue exhibit distinct spatial organisation: mGluRs are expressed in the anterior and posterior tongue, while Tas1r1/Tas1r3 is located mostly in the posterior part of the tongue (Yasumatsu et al. 2009).

Tas1r1/Tas1r3 responds to a range of amino acids in most mammals, but has a strong preference for glutamate and aspartate in humans (Nelson et al. 2002). The difference arises from structural differences between species in the extracellular Venus flytrap domain of the Tas1r1 subunit, which is responsible for amino acid recognition and binding (Toda et al. 2013). mGluR1 and mGluR4 are best known as receptors for L-glutamate, as suggested in the name, but recent findings in taste research have revealed a more diverse range of agonists, including L-amino acids arginine and serine (Choudhuri et al. 2016).

A characteristic feature of Tas1r1/Tas1r3 is that responses to amino acids via this receptor are enhanced in presence of nucleoside monophosphates, especially IMP, which is a compound of umami taste. IMP increases the

amplitude of the response in a concentration-dependent manner (in the range of 0.1–10 mM) by binding to the Tas1r1 subunit to stabilize the closed conformation of the receptor once an amino acid has bound to the Venus flytrap domain (Zhang et al. 2008, Yasumatsu et al. 2012). IMP alone does not elicit a response in cultured cells expressing Tas1r1/Tas1r3; neither does it have an amplifying effect on responses mediated by mGluRs (Yasumatsu et al. 2012) or the sweet taste receptor Tas1r2/Tas1r3 (Nelson et al. 2002).

As previous work has begun to draw the parallel between tanycyte and taste receptor cell signalling (Benford et al. 2017), the aim of this set of experiments was to test if the umami taste receptors Tas1r1/Tas1r3, mGluR1 and mGluR4 were responsible for tanycyte amino acid sensing.

Most results presented in this chapter have been published as part of Lazutkaite *et al.*, 2017.

3.2. Methods

3.2.1. Animals and brain slices

For Ca^{2+} imaging, male Sprague-Dawley rats (13–21 days old), male and female C57BL/6J mice (4 to 18 weeks old), and male and female *Tas1r1*-null mice (5 to 15 weeks old, kindly provided by the Meyerhof lab, described in Voigt et al., 2015) were humanely killed in accordance with the UK Animals (Scientific Procedures) Act 1986. The following procedure was the same as described in 2.2.1. IMP experiments were performed on rat tissue; the rest of the data were collected from mice.

Brain samples for *Tas1r1* expression imaging were obtained from the German Institute of Human Nutrition Potsdam-Rehbruecke. The frozen tissue of Tas1r1-*Cre/eR26-tauGFP* mice that expressed GFP at the site of *Tas1r1* (Foster et al. 2013, Voigt et al. 2015) was cut at 35 µm using a Bright OTF 5000 cryostat and mounted on slides with VECTASHIELD (Vector Laboratories, US) containing DAPI.

For immunohistochemistry, male C57BL/6J and *Tas1R1*-null mice were put in deep anaesthesia using Isoflurane, and cardiac perfusion with 4%

paraformaldehyde (PFA) was performed before dissecting the brain. The tissue was further immersion fixed for 24 hours in 4% PFA and transferred to phosphate-buffered solution (PBS). Slices were cut in the region of interest at 100 µm using a vibrating blade microtome.

3.2.2. Solutions

The solutions used in these experiments were the same as described in 2.2.2, with the addition of 200 µM 4-CPG (Tocris Bioscience, UK), 0.5–1 mM IMP (Sigma-Aldrich, UK), and 200 µM MAP4 (Tocris Bioscience, UK). 4-CPG and MAP4 were administered via bath application through the flow chamber for 15 minutes without imaging. IMP was applied through the flow system for 3–5 minutes to ensure it was available at the region of interest before the amino acid puffs.

4% PFA, PBS, and a blocking buffer (5% bovine serum albumin and 0.1% Triton in PBS) were used for immunohistochemistry.

3.2.3. Immunohistochemistry

Brain slices were arranged individually in a 24-well plate. They were rinsed with PBS 3 times (15 min. per rinse with gentle agitation) and placed in 250 µL of blocking buffer for 1 hour in room temperature with gentle agitation. The blocking buffer was then replaced with rabbit polyclonal anti-metabotropic glutamate receptor 4 antibody (ab53088, Abcam, UK) diluted to 1:250, 1:500, 1:1000 and 1:2000 in blocking buffer. The slices were incubated with the primary antibody overnight in 4°C. Next morning, the slices were rinsed with PBS (3×15 min. in room temperature with gentle agitation) and incubated with goat anti-rabbit Alexafluor 488 secondary antibody (Invitrogen, UK) diluted to 1:2000 in blocking buffer for 1 hour in room temperature with gentle agitation. The rinsing was repeated, and the slices were mounted on glass slides with Fluoroshield mounting medium (Sigma-Aldrich, UK), either with or without DAPI.

3.2.4. Imaging

Ca^{2+} imaging was performed as described in 2.2.3.

Tas1r1 and mGluR4 expression was visualised using a Leica SP5 confocal laser microscope. FIJI software was used for further analysis.

3.2.5. Data and statistical analysis

Data analysis was performed as described in 2.2.5. Statistical analysis for 4-CPG and MAP4 experiments was performed as described in 2.2.5. The G test was used to evaluate the significance of the change in the proportion of high responses ($\Delta F_{340}/F_{380} > 0.15$) in the IMP experiment.

In the *Tas1r1*-null versus wild type comparisons, a single animal was considered to be a replicate. On average recordings were made from 2 brain slices per animal for each amino acid and the results from each cell in the field of vision were averaged to give a single value per animal. Student's unpaired t test was used to compare the responses between wild type and *Tas1r1*-null animals. For all experiments involving *Tas1r1*-null animals, a power calculation was performed after gathering the initial data to determine the appropriate sample sizes. Randomization was not performed, as mouse litter sizes were small and all littermates were used.

3.3. Results

3.3.1. Inosine 5'-monophosphate increases tanycyte sensitivity to L-arginine

Tas1r1/Tas1r3 exhibits a strong synergistic response when amino acids are applied in presence of IMP. To test whether such synergism was present in tanycyte responses to amino acids, L-arginine was applied first in regular low-glucose aCSF, and then in low-glucose aCSF containing 0.5–1 mM IMP. The availability of IMP in the bathing medium resulted in an overall increase in the median response amplitude (Figure 3.1 A–C). Moreover, in presence of IMP, the number of cells exhibiting high ($\Delta F_{340}/F_{380} > 0.15$, threshold based on observation), rapidly rising responses increased from 14.8% to 40.9% (Figure 3.1 D).

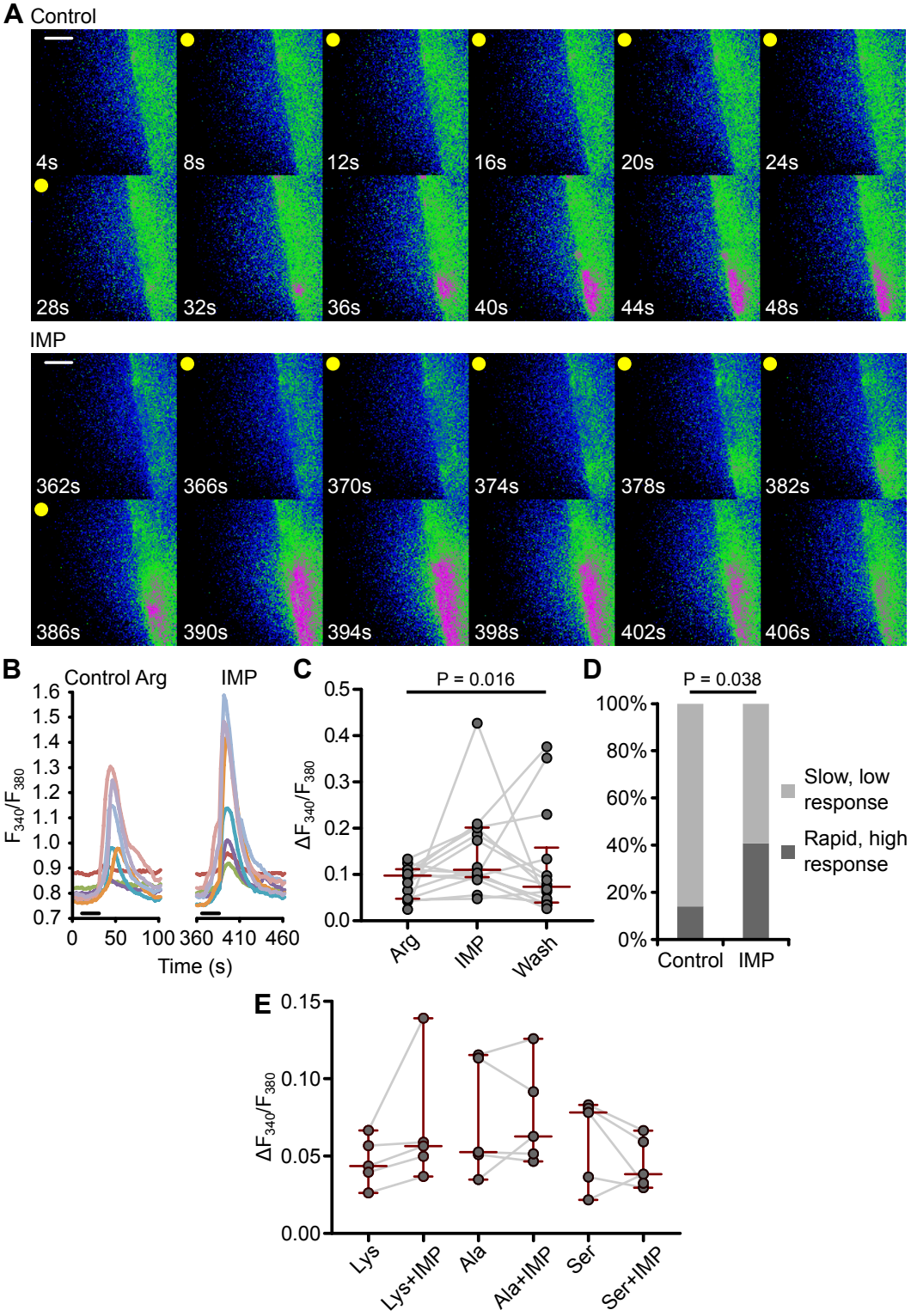

Figure 3.1. IMP enhances tanycyte responses to L-arginine. *(**A, B**) Montage and corresponding graph of ROI traces demonstrate the change of the response of one slice to a series of 300 ms puffs of 5 mM L-arginine in normal aCSF (Control) and after the addition of 0.5–1 mM IMP to the bathing medium (IMP). Scale bars 20 µm. (**C**) IMP increased tanycyte responses to L-arginine. P value given for Wilcoxon signed rank test. Black bars on the ROI traces represent the duration of amino acid puffing. (**D**) The proportion of cells exhibiting rapidly rising high responses (ΔF340/F380 > 0.15) increases from 14.1% to 40.9% with added IMP. P value given for G test. (**E**) Tanycytes exhibited a trend for increased responses to Lys with IMP (P = 0.063 Wilcoxon's signed rank test), but no changes in responses to Ala or Ser. Data for this graph was collected during my MSc.*

A previous experiment with IMP presented in my MSc dissertation also included Lys, Ala and Ser. Tanycyte responses to Lys demonstrated a trend for enhancement by IMP (P = 0.063, n = 5 Wilcoxon's signed rank test), whereas there was little to no change in the amplitude of the responses to Ala or Ser (Figure 3.1 E).

3.3.2. Tas1r1 is expressed in mouse tanycytes

In taste buds, multiple receptors are involved in the detection of amino acids. To confirm the presence of Tas1r1/Tas1r3 in tanycytes, mice modified using a binary genetic strategy were obtained from prof. Meyerhof's lab in Potsdam, Germany (Foster et al. 2013, Voigt et al. 2015). *Tas1r1-Cre* mice were bred with *Rosa26-tauGFP* reporter mice. In the offspring, the tauGFP reporter labelled cells normally expressing the *Tas1r1* gene. Confocal imaging of hypothalamic brain slices of these mice showed GFP expression in some, but not all tanycytes, and a number of unidentified neuronal or glial cells (Figure 3.2).

The fluorescence was dense in several areas: in the most anterior regions of the hypothalamus, the expression was scarce (Figure 3.2 A,B), but moving caudally towards the centre of 3V GFP was expressed in α1, α2, some β1 and β2 tanycytes, as well as a number of other cells on the outside of the ME, in the ARC, VMH, and some ependymal cells (Figure 3.2 C,D,G). In the posterior regions of 3V, GFP could be seen mostly in α2, β1 and β2 tanycytes and ependymal cells, but also unidentified cells in the ARC and VMH and a line of cells surrounding the ME (Figure 3.2 E,F).

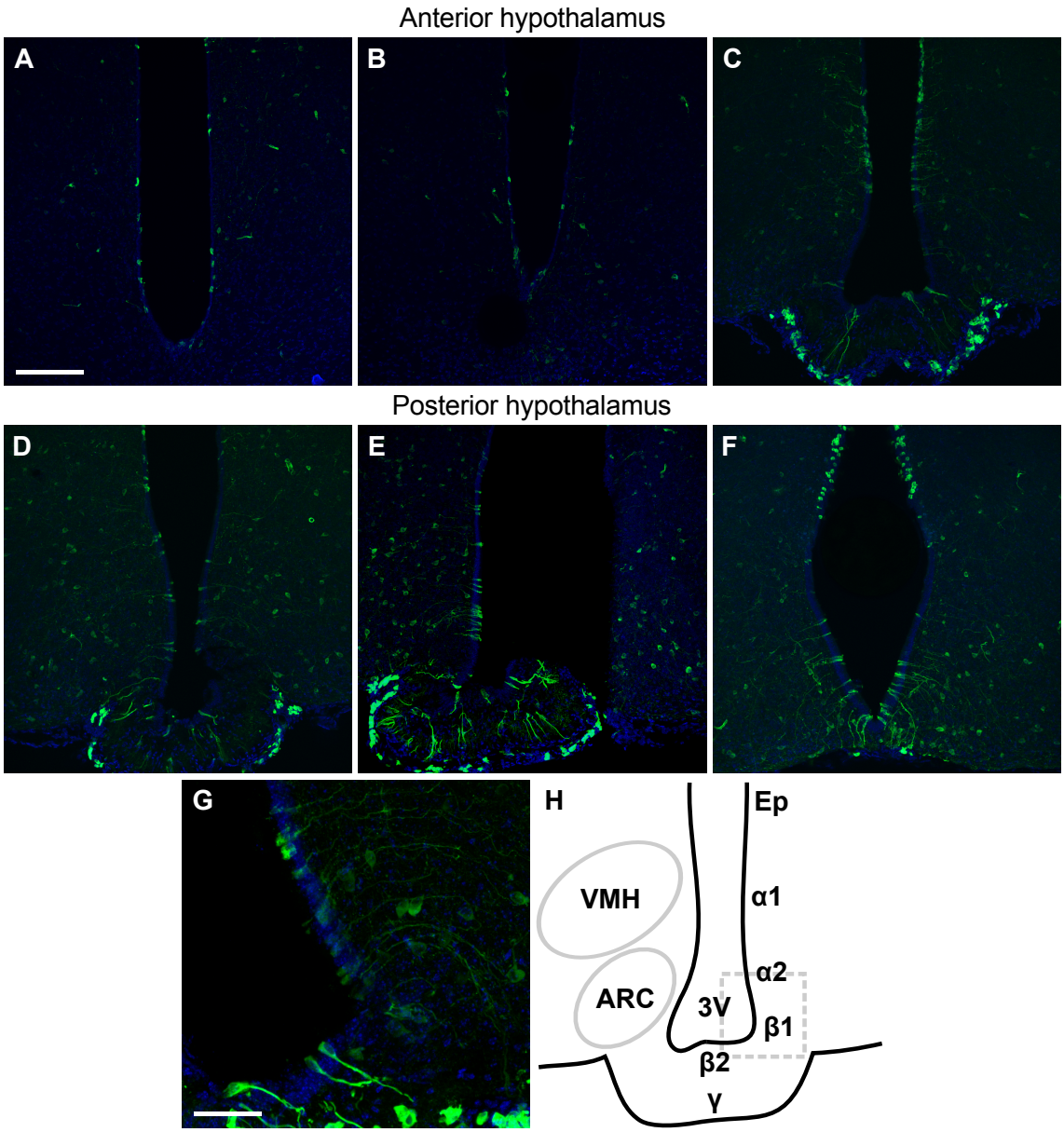

Figure 3.2. Tanycytes express Tas1r1. *(**A-G**) Hypothalamic tissue from Tas1r1-Cre/eRosa26-tauGFP mice shows that the Tas1r1 subunit is expressed in some, but not all hypothalamic tanycytes, as well as some neurons and ependymal cells. Little GFP is seen in the anterior parts of 3V (**A, B**), but the expression is consistent through the medial (**C, D**) and posterior (**E, F**) parts. Scale bar 200 µm. (**G**) A higher magnification image shows that cells expressing Tas1r1 in the wall of 3V exhibit tanycyte morphology. Scale bar 50 µm (**H**) Schematic representation of the area depicted in the fluorescence imaging. Grey dashed square represents the area shown in panel **G**. Greek letters indicate tanycyte populations. Ep, ependymal cells; VMH, ventromedial hypothalamic nucleus; ARC, arcuate nucleus; 3V, third ventricle.*

3.3.3. *Tas1r1*-null mice exhibit altered responses to L-amino acids

Ca^{2+} signalling in tanycytes of *Tas1r1*-null mice was examined to test whether their responses to amino acids were altered. These mice had a reporter construct consisting of a truncated barley lectin followed by an internal ribosome entry site and *mCherry* knocked into the *Tas1r1* locus (Voigt et al. 2012).

The *Tas1r1*-null mice (males and females analysed together) showed no difference in tanycyte responses to Ala (Figure 3.3 A). There was a striking variation in the responses to Lys and Arg, although the variances were consistent between the wild type and the *Tas1r1*-null groups. The responses to Lys were partially reduced, as well as the proportion of cells responding to Lys (P = 0.0264 and 0.0348, respectively, Student's t test), but there was no change in the responses to Arg (Figure 3.3 A).

To better understand the variability of the phenotype in the *Tas1r1*-null mice, the effects of age on tanycyte sensitivity to amino acids were analysed, but no relationship was found between age and amplitude of the responses to any of the tested amino acids within the experimental range (Figure 3.3 B). The responses were then separated by sex (Figure 3.3 C,D). In neither males nor females were the responses to Ala altered by deletion of the *Tas1r1* gene. The effect of *Tas1r1* deletion on Lys sensitivity was lower in the separate groups: a trend of reduction in the responses to Lys was observed in males (P = 0.0597 Student's t test); while in female *Tas1r1*-null mice, only the number of responsive cells decreased (P = 0.042 Student's t test; data not shown) without reducing the mean amplitude (Figure 3.3 C). The responses to Arg and the proportion of responding cells were strongly reduced in female *Tas1r1*-null mice (Figure 3.3 C). However, in male mice the responses to Arg were enhanced in the knock out compared to the wild type (Figure 3.3 D). This suggests that males can compensate for the loss of the *Tas1r1* gene (possibly via upregulation of another receptor) whereas female mice cannot. Interestingly, in wild type mice, tanycytes in females were more sensitive to Arg than those in males (P = 0.0024, Student's t test). This correlates with the differing effect of loss of the *Tas1r1* gene in the responses to Arg in tanycytes from male and

female mice. No similar findings have been reported before, and the biological significance of this difference between the sexes remains to be determined.

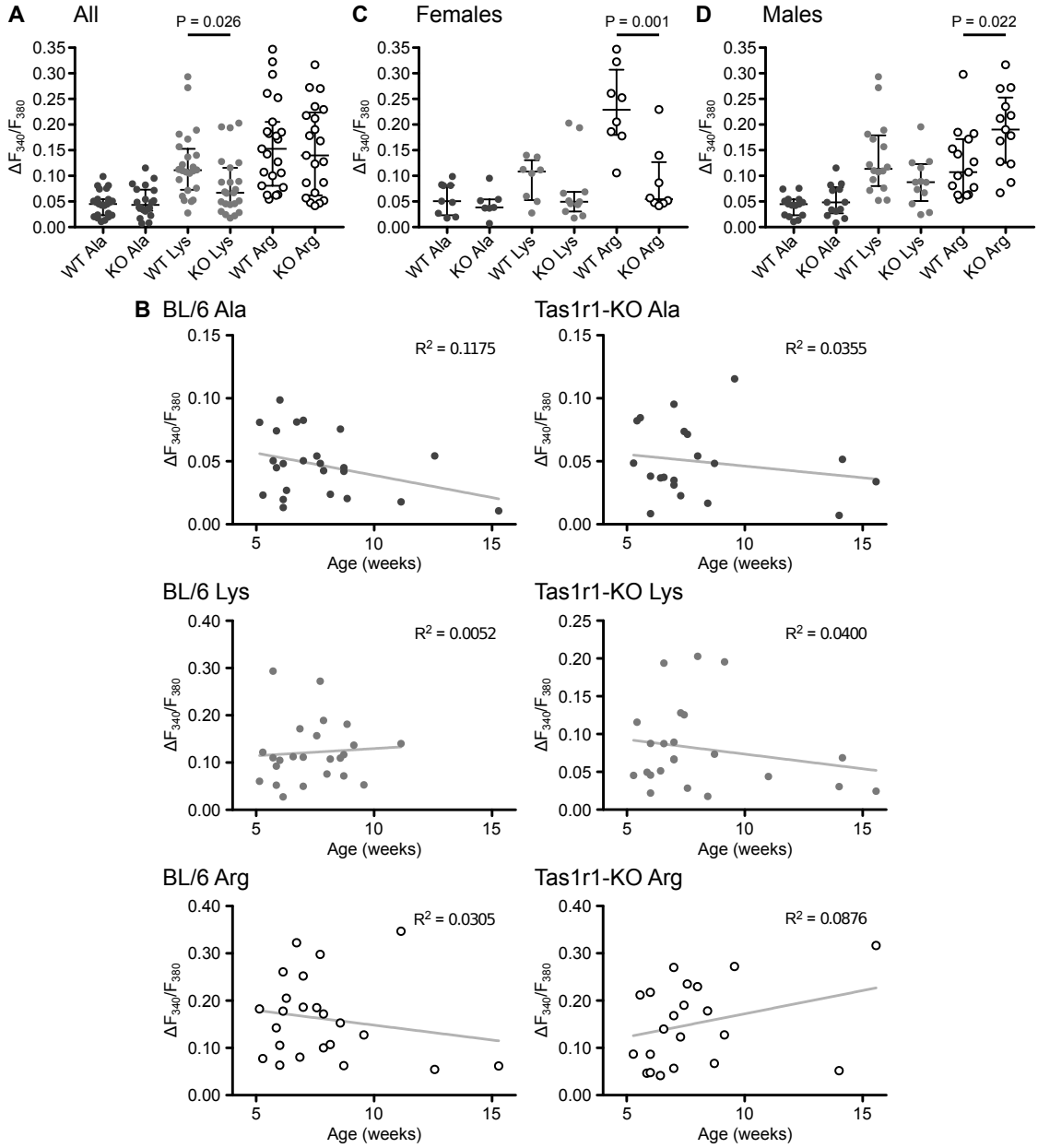

Figure 3.3. Tas1r1/Tas1r3 is involved in tanycyte amino acid sensing. *(**A**) Tas1r1-null mice showed no change in the responses to L-alanine or L-arginine but a reduction in the responses to L-lysine. (**B**) Tanycyte responses to amino acids in wild type and Tas1r1-null mice were not age-dependent between 5 and 16 weeks. (**C**) Female mice lacking Tas1r1 exhibited strongly reduced responses to L-arginine. (**D**) In male Tas1r1-null mice, tanycytes were more sensitive to L-arginine. P values given for Student's t test.*

3.3.4. mGluR4, but not mGluR1, mediates tanycyte responses to L-alanine and partially to L-lysine

Since Ala responses were unaffected by the deletion of *Tas1r1*, the next step was to investigate whether other known umami taste receptors, mGluR1 and mGluR4 (Maruyama et al. 2006), might be involved in tanycyte amino acid detection. Whereas the mGluR1 antagonist S-4-carboxyphenylglycine (4-CPG) (Sekiyama et al. 1996) did not have any effect on Arg, Ala or Lys responses (Figure 3.4 A), S-2-amino-2-methyl-4-phosphonobutanoic acid (MAP4), a specific mGluR4 antagonist (Johansen and Robinson 1995), greatly reduced tanycyte responses to Ala and partially reduced the responses to Lys (Figure 3.4 B–D). MAP4 had no effect on the responses to Arg.

3.3.5. Elimination of Tas1r1/Tas1r3 and mGluR4 is not sufficient to block all tanycyte responses to L-amino acids

To see whether a combination of blocking mGluR4 with MAP4 and *Tas1r1* gene deletion would have a more complete effect on amino acid responses, the effect of MAP4 in male *Tas1r1*-null mice was evaluated. The effect of MAP4 on tanycyte Ala sensitivity remained strong (Figure 3.4 E), but no change was observed in responses to either Lys or Arg. Any potential effect of MAP4 on responses to Lys may have been masked by the control Lys responses being lower in the *Tas1r1-null* mice. Nevertheless, if mGluR4 had been the only receptor mediating the responses to Lys in mice lacking the Tas1r1/Tas1r3 receptor, a more complete reduction of the responses under MAP4 would have been expected. It seems unlikely therefore that male mice compensate for the loss of the *Tas1r1* gene by upregulating mGluR4.

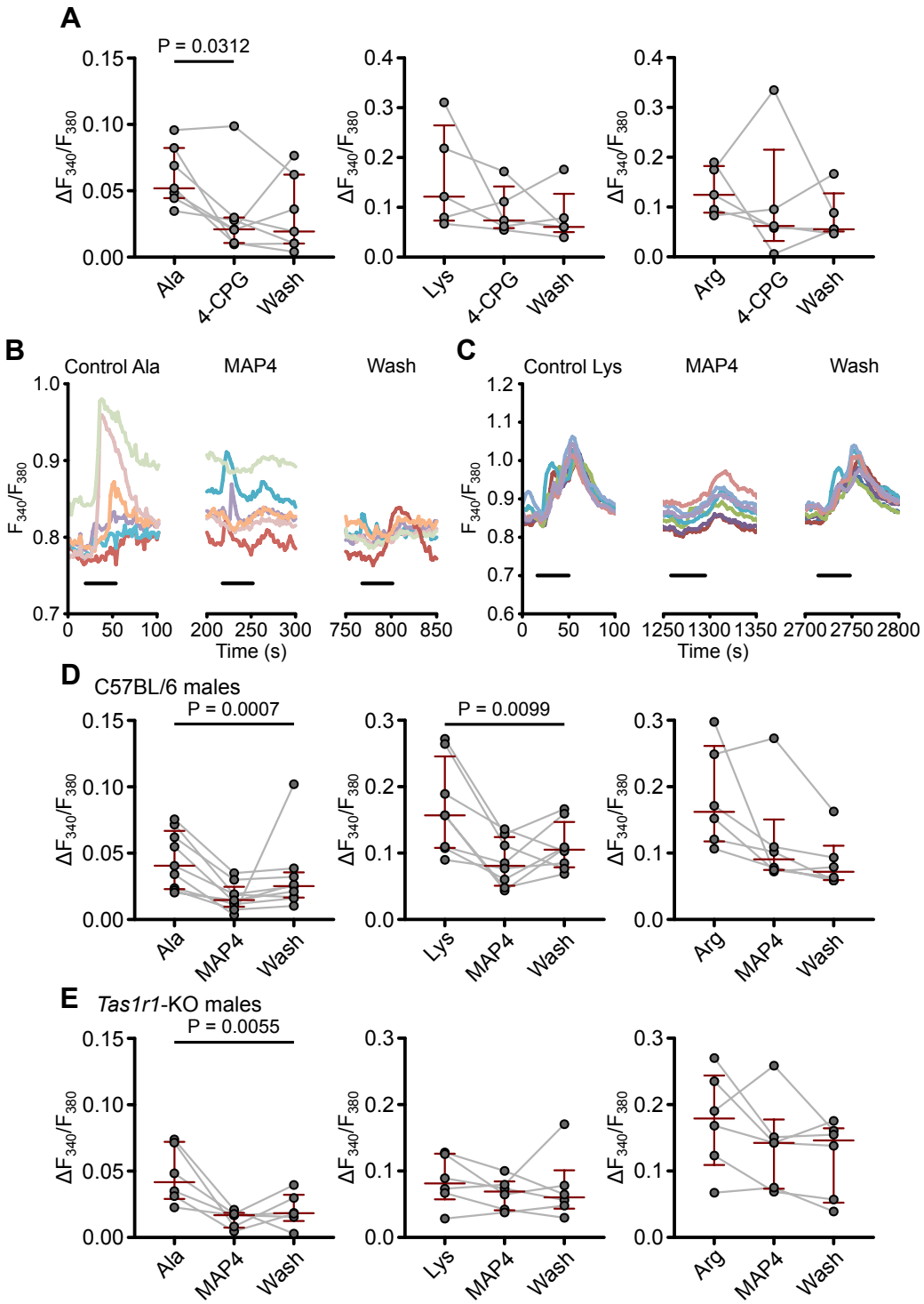

Figure 3.4. Tanycytes use mGluR4 to detect some L-amino acids. *(**A**) mGluR1 antagonist 4-CPG did not sufficiently block tanycyte responses to any of the tested amino acids. Some responses to L-alanine were reduced when 4-CPG was added (P = 0.0312 Wilcoxon's signed ranks test, n = 7 slices (from 4 animals)) but the recovery was inconsistent (P = 0.0854, Friedman test comparing Control Ala, 4-CPG and Wash). (**B, C**) Example ROI traces of the effects of the specific mGluR4 antagonist MAP4 on L-alanine and L-lysine. (**D**) MAP4 blocked tanycyte responses to L-alanine (P = 0.0007, Friedman test, n = 9 slices (from 6 animals)); the responses to L-lysine were also reduced (P = 0.0099, Friedman test, n = 8 slices (from 7 animals)). L-arginine responses were unaffected. (**E**) Using MAP4 on the Tas1r1-null mice did not eliminate all tanycyte responses to L-amino acids. The effect on L-alanine remained (P = 0.0055, Friedman test, n = 6 slices (from 6 animals)), while any effect on L-lysine was masked due to lower initial response (P = 0.7402, Friedman test, n = 6 slices (from 6 animals)). There was still no change in the responses to L-arginine.*

3.3.6. Mouse tanycytes express mGluR4

The Allen Mouse Brain Atlas shows expression of mGluR4, by *in situ* hybridization, in the ependymal layer surrounding 3V in the region of the median eminence (Lein et al. 2007). To further confirm the presence of mGluR4 in mouse tanycytes, immunohistochemical staining was performed on C57BL/6J and *Tas1r1*-null mouse brains.

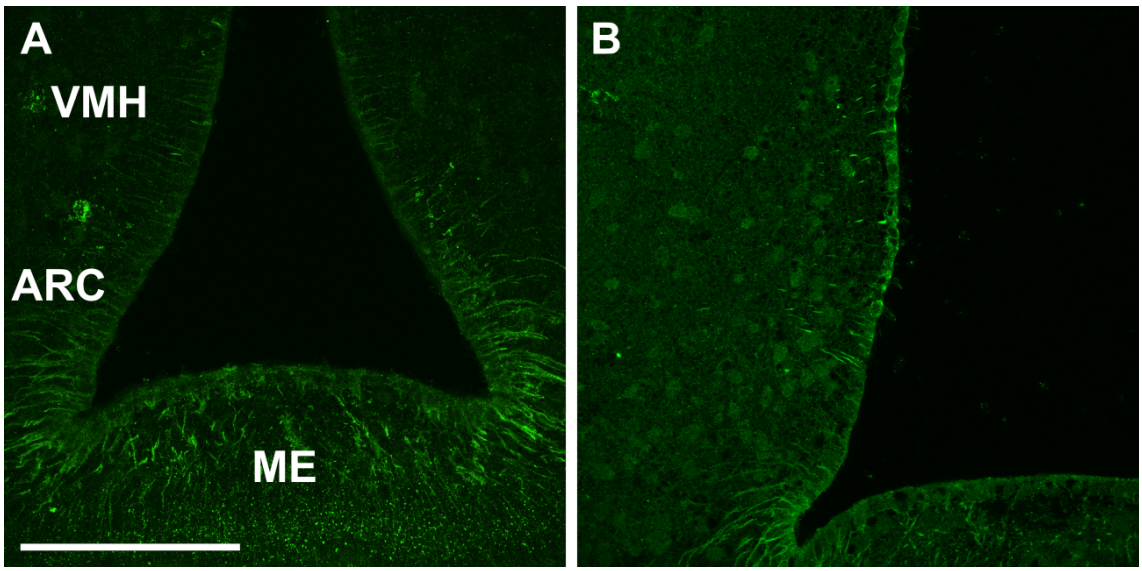

Figure 3.5. mGluR4 is expressed in hypothalamic tanycytes. *(**A**) mGluR4 labelling is visible in tanycyte processes throughout the median eminence, arcuate nucleus and ventromedial hypothalamic nucleus. (**B**) The intensity of the fluorescence is similar in Tas1r1-null mouse tissue, with additional labelling in tanycyte cell bodies, potentially extending to ependymal cells. Scale bar 200 µm. Abbreviations as in Figure 3.2.*

Expression of mGluR4-like immunoreactivity could be seen in a large number of cells in the hypothalamus, including the cells lining 3V that exhibited tanycyte and ependymal cell morphology (Figure 3.5 A). The fluorescence appeared similar in intensity in the *Tas1r1*-null brain (Figure 3.5 B).

There is no solid confirmation of the specificity for the primary antibody used in this experiment, although its use has been published in several papers (Wang et al. 2011, Betts et al. 2012, Chruścicka et al. 2014). Thus, while the immunohistochemical data alone would not be enough to confirm the presence of mGluR4 in mouse hypothalamus, it enhances the results of mGluR4 inhibition with MAP4, supporting the role of this receptor in tanycyte amino acid sensing.

3.4. Discussion

Tanycytes detect a range of L-amino acids, and they do so via at least two receptors – Tas1r1/Tas1r3 and mGluR4. According to the results from the previous chapter, the two receptors subsequently activate separate response pathways: the activation of Tas1r1/Tas1r3 leads to ATP release via Panx1, while mGluR4 triggers the opening of CalHM1 (Figure 3.6). While tanycyte glucosensing was long suspected and multiple glucose detection mechanisms have been demonstrated, tanycyte amino acid sensitivity has not been foreshadowed in literature. Therefore, the detection of amino acid concentration changes in the third ventricle by tanycytes is the first known non-neuronal

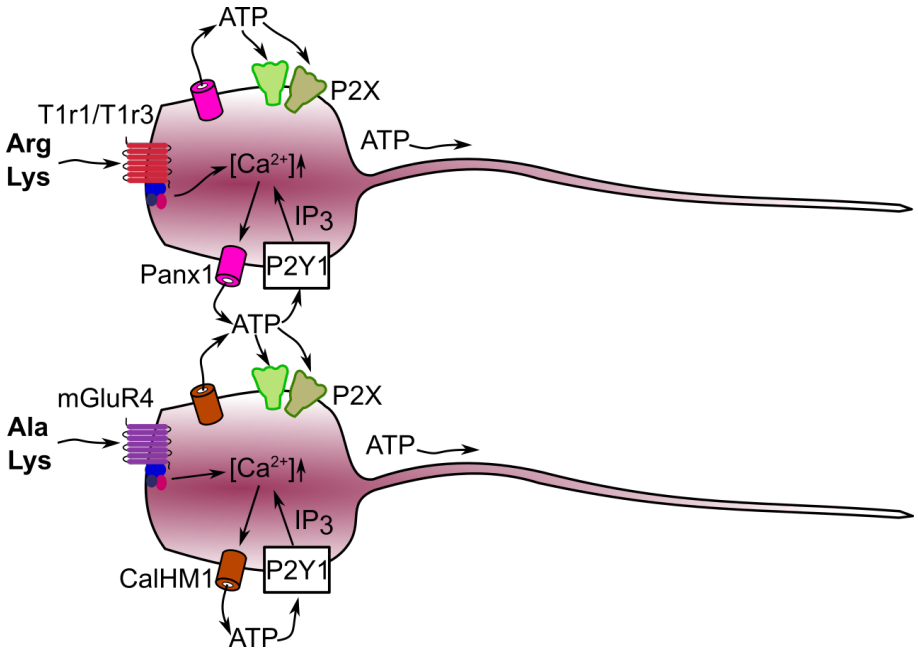

Figure 3.6. Proposed tanycyte amino acid signalling pathways. *Experimental evidence suggests that Tas1r1/Tas1r3 is required for the detection of L-arginine, while L-alanine is detected by mGluR4, and L-lysine can activate both receptors. The activation of the receptors increases intracellular Ca^{2+}, which opens membrane channels for ATP release. The ATP can then be picked up by nearby cells using P2 receptors, as well as travel down the tanycyte processes and into the parenchyma.*

mechanism of direct amino acid sensing in the brain.

3.4.1. Tanycytes use Tas1r1/Tas1r3 to detect L-arginine and L-lysine

This work shows that tanycytes functionally express Tas1r1/Tas1r3, and the loss of the Tas1r1 subunit of the receptor in mice is enough to affect tanycyte responses to Arg and Lys, depending on the sex of the mouse. Given that

Tas1r1 expression is sparse (Figure 3.2), the fact that the effects of knocking out this subunit were only partial is not entirely unexpected.

It is important to note that while a large number of tanycytes do indeed express Tas1r1, there are obvious gaps between cells expressing GFP in the confocal images (Figure 3.2). Tanycytes are often described as a heterogeneous population of cells, and it would not be surprising if individual tanycytes expressed only one of the amino acid receptors, or if there were sweet-specific, umami-specific and bitter-specific tanycytes. The latter organisation would reflect that of taste buds in the tongue, where each cell in a cluster can be activated by only one taste modality (Chandrashekar et al. 2006). The cell specificity could potentially be broken down further, with separate umami-sensitive tanycytes expressing different umami taste receptors. This arrangement would enable tanycytes to send very well-defined signals to dedicated neural populations as different mixtures of dietary amino acids would activate separate subsets of tanycytes, thus maximising the amount of information delivered by these cells.

3.4.2. Multiple receptors are required for tanycyte detection of L-amino acids

As the group III mGluR antagonist MAP4 strongly reduced all responses to Ala and partially blocked those to Lys, it is evident that mouse tanycytes also use mGluR4 to detect at least these two amino acids. This is further supported by the apparent expression of mGluR4 throughout the hypothalamus, including the wall of the third ventricle. The fact that multiple receptors are involved in the detection of amino acids reflects the current knowledge in umami taste research and suggests that tanycyte amino acid sensing is a physiologically important mechanism.

The nature and identity of the umami taste receptor has long been the subject of an on-going discussion between various groups investigating human and rodent taste receptors: while a significant body of evidence pointed towards Tas1r1/Tas1r3, knocking out the Tas1r3 subunit of this receptor in mice did not eliminate all responses to umami-tasting amino acids (Damak et al. 2003,

Maruyama et al. 2006). The current consensus is that additional receptors such as mGluR1 and mGluR4 can sense a range of amino acids in the taste receptor cells in the tongue, and these mechanisms can compensate for each other when one of the receptors is lost (Yasumatsu et al. 2012). Having different receptors for various amino acids (with some overlap) could also help identify the overall quality of the food, such that if all receptors were activated at once, that would mean the ingested food was nutritious and rich in a variety of amino acids.

Many studies using genetically modified animals to investigate metabolic challenges have resulted in a rescue of phenotype by upregulation of other genes or activation of alternative signalling pathways (Leroux et al. 2001, Castro-Chavez et al. 2003, Roncon-Albuquerque et al. 2008, Castillo et al. 2011). A possible explanation why some responses to amino acids were still observed in the *Tas1r1*-null mice when mGluR4 was blocked (see Figure 3.4 E) could be that due to the similarities between Tas1r1/Tas1r3 and Tas1r2/Tas1r3, the sweet taste receptor could adapt to function as a sensor for both glucose and some amino acids in rodents lacking the *Tas1r1* gene. While the potential of such an adaptation has not been widely discussed, Tas1r1/Tas1r3 and Tas1r2/Tas1r3 do exhibit species-specific sensitivity profiles; for example, hummingbird Tas1r1/Tas1r3 has evolved to detect sugar in plant nectars (Baldwin et al. 2014).

3.4.3. Physiological changes in amino acid levels in the third ventricle could potentially activate tanycytes *in vivo*

In the experiments described in this and previous chapter, large concentrations (up to 10 times the baseline concentration in hypothalamic CSF based on Choi *et al*., 1999) of single amino acids were applied acutely to the tanycyte layer. Such approach does not accurately replicate the events occurring in 3V after feeding, but similar tanycyte activity can be expected in vivo, even if individual amino acid concentrations are relatively low, due to the following reasons.

As mentioned before, IMP is an allosteric modulator of the Tas1r1/Tas1r3 receptor, but has no effects on other known amino acid receptors (Yasumatsu

et al. 2012). In these experiments IMP enhanced tanycyte responses to Arg, providing further support for the functional involvement of Tas1r1/Tas1r3 in tanycyte amino acid sensitivity. The concentrations of the two essential amino acids used in this study – Arg and Lys – only increase by a small amount after feeding (Choi et al. 1999), but the combination of multiple amino acids ingested at once and the potentiation of the Tas1r1/Tas1r3 receptor by IMP could be sufficient to evoke tanycyte signalling.

Conversely, the levels of alanine in both blood plasma and CSF vary greatly depending on food intake (Anderson et al. 1994). This, combined with the prevalence of alanine in cereals and other foods that make up rodent diets, makes alanine a likely candidate for a generic signal of protein consumption for tanycytes. If this is the case, the proposed tanycyte dual amino acid receptor model could use the amino acid information in two different ways: transient alanine signals would reflect feeding, while a more tonic essential amino acid signal would be used to monitor overall nutritive health.

Other similar, nutritionally relevant amino acids should be investigated to check whether tanycyte responses exhibit a cumulative increase, or a certain few amino acids are used to regulate the entire tanycyte signalling mechanism.

3.4.4. Male and female mice adapt differently to the loss of *Tas1r1*

Both male and female mice were used in the *Tas1r1*-null experiment to minimise the number of unused animals culled following the “3Rs” stated in the Animals (Scientific Procedures) Act 1986. However, as the number of animals used for the study increased, differences between male and female tanycyte responses to amino acids started to emerge: firstly, wild type female mice exhibited higher responses to Arg than wild type males; secondly, the loss of *Tas1r1* reduced the responses to Arg in females but had the opposite effect in males (Figure 3.3 C,D). This suggests that male and female mice possess different adaptive mechanisms for genetic challenges in tanycyte amino acid detection.

Recent literature on energy homeostasis in male and female mice supports the sex differences in the adaptation to the loss of Tas1r1: further differences have

been observed in POMC expression in response to a high-fat diet (Plum et al. 2006) and the effects of changes in diet on hypothalamic neurogenesis (Lee et al. 2014, Bless et al. 2016). Moreover, POMC-derived peptides α-MSH and desacetyl-α-MSH affect male and female mice differently – male mice lacking both peptides are as sensitive to reintroduction of desacetyl-α-MSH as α-MSH, while female mice only exhibit weight loss when administered α-MSH (Mountjoy et al. 2017).

The oestrous cycle of female mice was not taken into account in this experiment, but, since oestrogen receptors are expressed widely in the hypothalamus and are known to influence energy homeostasis (Iwasa et al. 2017) and tanycyte morphology (De Seranno et al. 2010), it is possible that if the study was repeated using animals in any one phase of the cycle, the results could be slightly different. The amount of circulating oestrogen affecting tanycyte responses to amino acids is a tempting explanation for the outliers in the *Tas1r1*-null responses to Lys and Arg (Figure 3.3 C); however, male *Tas1r1*-null responses also have a similar distribution without any known differences in oestrogen.

In conclusion, hypothalamic tanycytes use a taste receptor system similar to that in the tongue and comprised of Tas1r1/Tas1r3, mGluR4 and potentially another receptor to detect changes in L-amino acid concentrations within 3V. The complexity of the system, as well as the rescue of Arg responses in male mice lacking the Tas1r1 subunit of the Tas1r1/Tas1r3 receptor, reinforce the prospective significance of tanycyte umami taste signalling. Moreover, tanycyte signalling is different in male and female mice, which should be taken into account in further research, especially with regards to clinical applications of any future findings.

4. Diet-related changes in feeding, metabolism and tanycyte sensitivity to amino acids

4.1. Introduction

Hypothalamic tanycytes can sense changes in nutrient concentrations in the CSF of the third ventricle using a range of taste receptors. The position of tanycytes close to the neuronal circuits in the ARC and VMH that regulate feeding and energy expenditure, combined with the multitude of nutrient detection pathways in tanycytes, indicate a key role for these cells in energy homeostasis. This set of experiments aimed to investigate the physiological significance of tanycyte amino acid signalling.

The regulation of energy homeostasis depends closely on the qualities of consumed food. In addition to caloric intake-dependent release of gut peptides, the composition of one's diet can affect feeding and energy expenditure via multiple central pathways. For example, both high-fat and low-protein diets can impair neurogenesis in the hypothalamus (Lee et al. 2012, Lee et al. 2014). HFD also leads to neuroinflammation, apoptosis of hypothalamic neurons, and reduction in synaptic inputs in the ARC and LH, leading to increased body mass and resistance to leptin and insulin (Moraes et al. 2009). POMC neurons of mice with HFD-induced obesity exhibit higher intracellular Ca^{2+}, hyperpolarization, reduced excitability and low spontaneous activity (Paeger et al. 2017). Moreover, rat pups exposed to maternal HFD have increased levels of orexigenic peptides such as galanin, orx and MCH, and newly generated neurons in the 3V of these pups are more likely to be incorporated into the orexigenic networks (Chang et al. 2008). HFD also alters circadian rhythms, changing the diurnal patterns of circulating metabolic hormones leptin and insulin, nutrients such as glucose and free fatty acids, and hypothalamic peptides POMC, CART, NPY, AgRP and orx (Kohsaka et al. 2007). Hypothalamic tanycytes exhibit an increase in lipid droplets in animals on chronic HFD (Hofmann et al. 2017).

On the other hand, HFD-fed mice supplemented with L-leucine exhibit decreased diet-induced obesity due to higher energy expenditure through

thermogenesis (Zhang et al. 2007). Leu also improves glucose metabolism and reduces HFD-induced insulin resistance (Zhang et al. 2007). The effects of Leu supplementation can be traced back to the hypothalamus, where Leu potently activates the mTOR pathway in hypothalamic NPY/AgRP neurons, reducing the production of orexigenic peptides (Cota et al. 2006). A recent study has shown that an additional mechanism is involved in hypothalamic Leu sensing, as blocking mTOR targets does not suppress Leu signalling in POMC neurons; interestingly, inhibiting previously suggested Leu transporters, K_{ATP} and GPCR pathways also failed to reduce Leu detection, suggesting that a yet unknown receptor is involved in its anorexigenic effects (Heeley et al. 2018).

High-protein (HP) diets tend to reduce food intake and increase satiety, although the effects are short-term as partial adaptation occurs (Bensaïd et al. 2003). In humans, ingestion of protein also stimulates thermogenesis and overall energy expenditure more effectively than carbohydrates or fat (Steiniger et al. 1987, Mikkelsen et al. 2000). While one study suggested that animals monitor their protein intake by detecting L-glutamate in food via both taste and gut signalling (Kondoh et al. 2009), glutamate supplementation alone did not result in reduced food intake and body weight (Boutry et al. 2011).

In studies utilising HP diets to investigate their effects on food intake and metabolism, the composition of the protein can influence the outcome: for example, a 55% whey protein diet is more efficient in reducing feeding than a 55% whole milk protein diet (Pichon et al. 2008). A difference between the effects of whey, soy protein and egg albumen on subsequent meal size was also demonstrated in human subjects (Anderson et al. 2004). Oral, intraperitoneal and intracerebroventricular administration of L-arginine has been shown to reduce food intake in male rodents (Alamshah et al. 2016). L-phenylalanine also reduces food intake via CaSR-mediated gut peptide release (Alamshah et al. 2017). This suggests that the amino acid composition of the food, rather than total protein intake, can determine the outcome of dietary interventions.

As food intake leads to an increase of amino acids in the blood plasma and subsequently in the CSF (Anderson et al. 1994), and hypothalamic tanycytes

are directly exposed to these changes, it was interesting to determine whether tanycyte signalling could be affected by the amino acid content of the diet, and if the effects would be reflected in rodent food choice and metabolism.

4.2. Methods

4.2.1. Animals and tissue preparation

For Ca^{2+} imaging experiments, male Sprague-Dawley rats (21–28 days old) were fasted for 15 hours (ensuring weight loss under 15%), given one of the amino acid imbalanced (IMB) diets (see 4.2.2) for 22 hours, or given standard laboratory rodent chow (EURodent diet 14%, LabDiet, US) before being humanely killed in accordance with the UK Animals (Scientific Procedures) Act 1986. The following procedure was the same as described in 2.2.1.

For refeeding after short-term fasting experiments, 12 male Sprague-Dawley rats (22-24 days old on day 1 of the experiment) were singly housed in normal conditions with free access to normal laboratory animal chow and water. The chow was removed from 6 cages at 1600 on the first day of the experiment; at 0800 the following morning 50 g of chow was placed directly inside each cage, and the animals were provided with drinking water supplemented with either 50 mM Ala or 29 mM Pro. The amount of food remaining in the cage was measured 4, 8, and 24 hours later. The rats were then given 32 hours to recover with *ad libitum* chow and normal drinking water. The experiment was repeated with reversed groups: animals that had previously been fasted received *ad libitum* chow, while animals that were in the control group were now fasted.

For amino acid supplement preference experiments, male and female C57BL6/J or *Tas1r1*-null mice (6–9 weeks old, housed in same sex and same genotype pairs) were given the arginine and lysine deficient diet (RK-; see 4.2.2) or normal chow for 24 hours and then moved to new cages to monitor their food and amino acid supplement intake for 24 hours. After that they were given 6 days to rest in their home cages with *ad libitum* chow before repeating the experiment with reverse diets: mice that had been given RK- in the first round received chow in the second round, and vice versa. They were then kept

in their home cages for another 6 days (for experimental timeline see Figure 4.4 A). All mice were weighed every day during the active parts of the experiment, and every 2 days during the rest periods. All cage and diet changes, as well as animal weighing, were performed in the first half of the light phase.

For experiments using metabolic cages – the Comprehensive Laboratory Animal Monitoring System (CLAMS) – 16 male (experiment 1) and 16 female (experiment 2) mice heterozygous for the *NrCAM* gene (*NrCAM*$^{+/-}$; (Sakurai et al. 2001), 10–14 weeks old at the start of the experiment) were used. The mice were individually housed for 1 week before the first two groups (n = 4 mice per group) were transferred to the metabolic cages to measure daily activity, food intake and energy expenditure. In the first CLAMS experiment, the first group (RK1) was provided with ground RK- diet, while the second group (RK2) received ground high protein chow (Teklad Global 18% Protein Rodent Diet, Harland Teklad, UK) for 24 hours. For the following 24 hours, both groups received ground alanine enhanced (A+) diet. They were then returned to their home cages, and the third (A1) and fourth (A2) group went into the CLAMS, where A1 mice were given ground alanine deficient (A-) diet, and A2 received ground chow for 24 hours. After that, both groups were provided with the A+ diet for 24 hours before returning to their home cages. One week after the start of the first trial, the experiments were repeated with reversed diets for the first 24 hours in the CLAMS: RK1 received chow, RK2 received RK-, A1 received chow, and A2 received A-; all mice were given A+ for the following 24 hours (timeline of experiment shown in Figure 4.6 A and on a smaller scale in Figure 4.8 A). At the end of the last trial, 8 more age-, sex- and weight-matched wild type mice from the same colony were added to the experiment and all mice were reassigned into new groups to be given RK-, A-, A+ or chow for 22 hours. They were then culled and brain samples were collected for future analysis.

The design and timetable of the second CLAMS experiment mimicked the first one, except female mice and a matched control diet were used this time.

4.2.2. Diet

The IMB diets – arginine and lysine deficient, alanine deficient and alanine enhanced – were obtained from Special Diets Services, UK. The formulation of the diets was based on standard laboratory mouse chow (Rat and Mouse No.1 Maintenance Autoclavable diet, Special Diets Services, UK). RK- had no Arg in the formulation (0.7% in standard chow) and Lys was reduced to 0.27% (0.75% in chow). A- had Ala removed from the formulation (0.87% in chow). A+ had Ala supplemented to achieve 1.74%. All IMB diets were balanced on a carbohydrate source. The IMB diets and the chow diets had similar Atwater energy density (3.1–3.3 kcal/g).

All diets were used in pellet form in Ca^{2+} imaging and 2-bottle experiments and in powder form in CLAMS experiments.

Three different control diets were used in these experiments. For the Ca^{2+} imaging, refeeding and 2-bottle experiments, the EURodent 14% diet was used. In the first CLAMS experiment, mice received the TEKLAD Global 18% diet. A nutrient- and calorie-matched control diet (C2) was used in the second CLAMS experiment instead of standard chow. The C2 diet was formulated by grinding the A+ and A- diets and mixing them at a 1:1 ratio to achieve Ala level of approximately 0.95% while keeping the caloric content, fat, protein and carbohydrate proportions, fibre content, smell and texture the same as those of the IMB diets.

A 50 mM Ala solution and a 25 mM Arg + 25 mM Lys solution were presented to the animals during the amino acid supplement preference experiment. Blackcurrant flavouring (10% by Sainsbury's No Added Sugar Double Strength Blackcurrant Squash with sucralose) was added to the solutions to mask the flavour of the amino acids. The water in drinking bottles was also flavoured to match the taste.

4.2.3. Experimental cages

In the amino acid supplement preference experiment, custom-made drinking tubes were used in the food and drink monitoring cages to present the amino

acid solutions. Each drinking tube consisted of a 25 ml Fisherbrand disposable polystyrene serological pipette (Fisher, UK) with a widened opening connected to a Tecniplast AISI 316 bottle lid with a sipper tube via a 165 mm Tygon silicone tube (inside diameter 4.8 mm, outside diameter 7.9 mm; Saint-Gobain Performance Plastics, US) and the middle section of a TipOne 1 ml filter tip (Starlab, UK). A stopper was fitted at the wide end of the pipette. The silicone tube was removed when filling the pipette with an amino acid solution, then replaced immediately and flicked gently to remove any bubbles. The filled drinking tube was then fixed in a retort stand, and the sipper tube was slotted between the bars of a stainless-steel wire cage lid. Two drinking tubes (Ala and Arg+Lys) plus a standard drinking bottle with flavoured water were provided for each cage. 30 g of RK- or chow pellets were placed directly into the cages. There were 6 cages with 2 mice per cage.

The CLAMS experiments were performed at Queen's Medical Centre, School of Life Sciences, University of Nottingham, with training and assistance from Prof. Francis Ebling and Dr. Jo Lewis. The experimental system was obtained from Linton Instrumentation, Linton, UK, and manufactured by Columbus Instruments, Columbus, OH, US. The CLAMS cages consisted of a centre feeder cage on scales to monitor food intake, Oxymax open circuit indirect calorimeter, and infrared photocells to detect animal motion.

The parameters monitored in the CLAMS were total food intake, feeding bout frequency and size, oxygen consumption (VO_2), carbon dioxide production (VCO_2), respiratory exchange ratio (RER), heat production and activity levels. The data were recorded using Oxymax software (Columbus Instruments, US).

4.2.4. Imaging

Ca^{2+} imaging was performed as described in 2.2.3. The experimental solutions used were the same as in 2.2.2.

4.2.5. Data and statistical analysis

Ca^{2+} imaging data analysis was performed as described in 2.2.5. The proportion of cells responding was determined using a threshold of $\Delta F_{340}/F_{380} \geq 0.02$

(based on changes in Ca^{2+} normally occurring in the baseline) for each cell and calculating the number of responsive and unresponsive cells for each animal. These numbers were then compared between different dietary conditions using Fisher's exact test. Tanycyte response amplitudes were compared as described in 2.2.5.

The food, water and amino acid intake data from the 2-bottle preference test were collected per cage (2 mice) rather than per animal. Amino acid intake in ml was then converted to mg calculated using the molarity of each solution. Paired Student's t tests were performed on all data using Graphpad Prism 7; each animal was considered a replicate and went through both the RK- and control treatments. Mann-Whitney U test was used to compare wild type and Tas1r1-KO mouse results in this experiment as some of the data had unequal variances.

The data from the CLAMS were divided into 1-hour subsets and averages for each hour were calculated for the metabolic data. These averages were then used for further analysis of the parameters in the dark and light phases. Total food intake, feeding time and feeding bout frequency were calculated in 3-hour bins, which were then averaged for the dark and light phases. Fat oxidation (Fox), carbohydrate oxidation (CHOx) and energy expenditure (EE) were calculated using the following formulae (Frayn 1983):

$$Fox = (1.67 \times VO_2) - (1.67 \times VCO_2)$$
$$CHOx = (4.55 \times VO_2) - (3.2 \times VCO_2)$$
$$EE = (15.9 \times VO_2) + (5.2 \times VCO_2)$$

The data were plotted and statistical analyses were performed using Graphpad Prism 7. Paired statistical analyses (Wilcoxon's matched pairs test or Student's paired t test) were performed where the same animal was exposed to different treatments.

4.3. Results

4.3.1. Short-term fasting increases tanycyte sensitivity to L-alanine

To determine the effects of fasting on tanycyte responses to amino acids, male Sprague-Dawley rats were fasted overnight (15 hours) prior to tissue collection. The rats lost on average 9.25% (SEM 1.36%, n = 8) of their initial weight.

While there was a hint of increased tanycyte Ca^{2+} responses to Ala after fasting, there were no significant differences between the amplitudes of the responses to Ala, Arg or Lys in fasted and fed animals (Figure 4.1 A–C). However, the variance of the responses to Ala increased in the fasted animals, which may mean that cells which would normally not respond to Ala were producing small Ca^{2+} waves, while cells that would be sensitive to Ala under normal feeding conditions had increased responses. Alternatively, as the recordings were made in slices between approximately Bregma -2.3 mm and Bregma -3.5 mm, the changes in the response variance could reflect area-dependent up- and down-regulation of tanycyte sensitivity to Ala; however, the slices for Ca^{2+} imaging were chosen at random, and this hypothesis could not be tested *post hoc*.

Further analysis revealed that fasting increased the proportion of tanycytes responding to Ala from 67.3% to 90.3% (Figure 4.1 D). No such changes were observed in the responses to Arg or Lys (Figure 4.1 E,F), suggesting that fasting induced an upregulation of tanycyte receptors sensitive to Ala (most likely mGluR4) or their downstream targets, but not Tas1r1/Tas1r3.

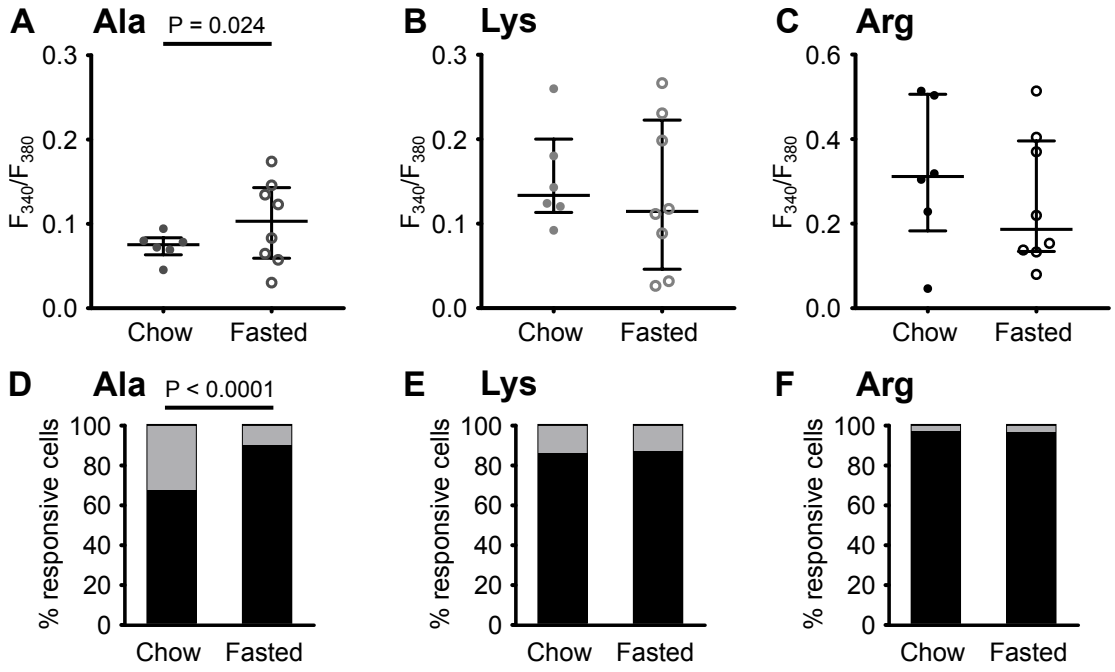

Figure 4.1. Fasting increases tanycyte sensitivity to L-alanine. *(**A, D**) An overnight fast affected the variance of tanycyte responses to L-alanine (F test for variances) and increased the proportion of responsive cells (Fisher's exact test). Fasting did not affect the responses to L-lysine (**B, E**) or L-arginine (**C, F**).*

4.3.2. Oral L-alanine supplementation does not alter food intake after short-term fasting

If the fasting-induced increase in tanycyte Ala sensitivity was a part of the regulatory mechanism for energy homeostasis, this would be reflected in food intake or energy expenditure. Thus, another experiment was performed to evaluate the potential role of Ala in re-feeding after an overnight fast.

Rats were single housed and received ad libitum laboratory chow. At 1600 on the first day of the experiment, food was removed from half of the cages, and 50 g of food was returned at 0800 the following morning. The rats were given drinking water supplemented with 50 mM Ala or 29 mM Pro. Food intake was measured after 4, 8 and 24 hours. Pro was chosen as a control amino acid due to its inability to activate tanycytes and no reported effects on food intake.

Rats lost on average 11.93 ± 0.82% of their bodyweight during the 16-hour fast; control animals gained 7.65 ± 0.76% during the same period of time. The weight lost during fasting was partially compensated for in the 24 hours of refeeding, when fasted rats gained 20.97 ± 1.31% bodyweight, compared to 8.94 ± 0.52% in fed rats. Animals that received Ala and Pro in their drinking water gained a

similar amount of weight in both fed and fasted groups. Total weight change in grams is shown in Figure 4.2 A and B.

The amount of food eaten in the first 4 hours after reintroduction of food was unsurprisingly higher in fasted than fed rats, but there was no difference between the fasted groups supplemented with Ala and Pro (Figure 4.2 C). The results were similar after 8 hours as both time points were in the light phase (Figure 4.2 D).

After 24 hours of refeeding, the fed animals had consumed as much food as the fasted groups. Ala supplementation still had no effect on food intake in fasted rats, while fed rats ate slightly (yet insignificantly) more with Ala than Pro (Figure 4.2 E).

The amount of Ala or Pro consumed with drinking water could not be measured in this experiment, making it likely that the Ala dose was insufficient to have an effect on feeding. A direct method of Ala administration (e.g. intragastric or ICV) would yield more accurate results.

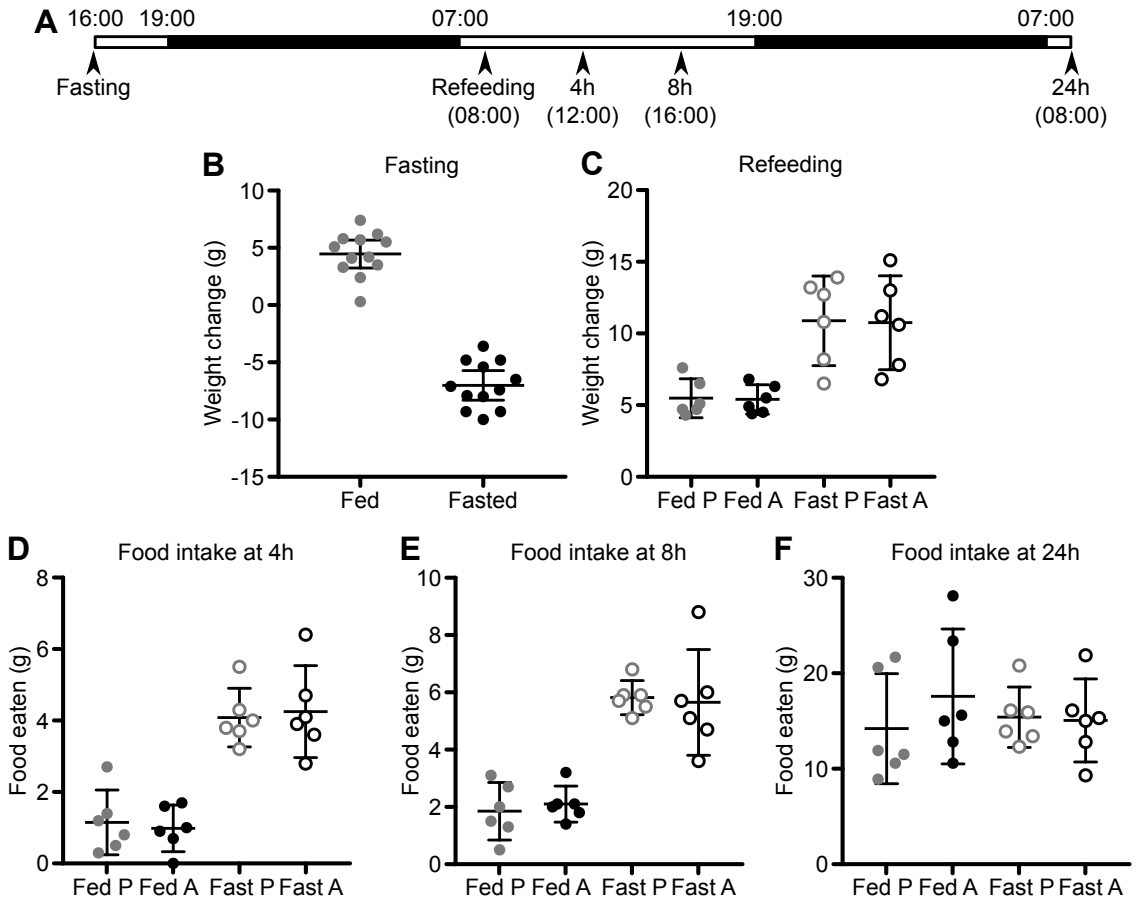

Figure 4.2. L-alanine supplementation in water does not affect food intake after feeding. *(**A**) Fasted male rats lost on average 7 grams of weight during the 16 hour fast, while their fed littermates gained around 4.5 grams. (**B**) Ala and Pro supplemented in drinking water did not change the amount of weight gained during the 24 hours of refeeding. (**C–E**) Food intake of fed and fasted rats supplemented with Ala or Pro after 4, 8 and 24 hours of refeeding. Both fasted groups consumed more food at the start of the refeeding period (after 4 and 8 hours), which was in the light phase, but total food intake after 24 hours was not different between any of the groups.*

4.3.3. Amino acid-imbalanced diet changes tanycyte sensitivity to amino acids

To investigate if amino acid content in the diet could influence tanycyte sensitivity, male Sprague-Dawley rats were given an Arg and Lys deficient diet, Ala deficient diet, or kept on normal chow for 22 hours before being culled for tissue collection.

No differences in the response amplitudes to any of the three tested amino acids were observed between chow and either of the amino acid-imbalanced diets (Figure 4.3 A–C), although tanycytes from rats fed the A- diet exhibited higher responses to Arg than those treated with the RK- diet. However, due to the high variance in the control responses and the lack of difference between

the A- and Chow groups, these results should be interpreted with caution as the RK- and A- experiments were run several months apart, so the effects could be skewed by external factors such as seasonal changes, possible switch in animal suppliers, or subtle differences in the laboratory conditions during brain slice incubation and imaging.

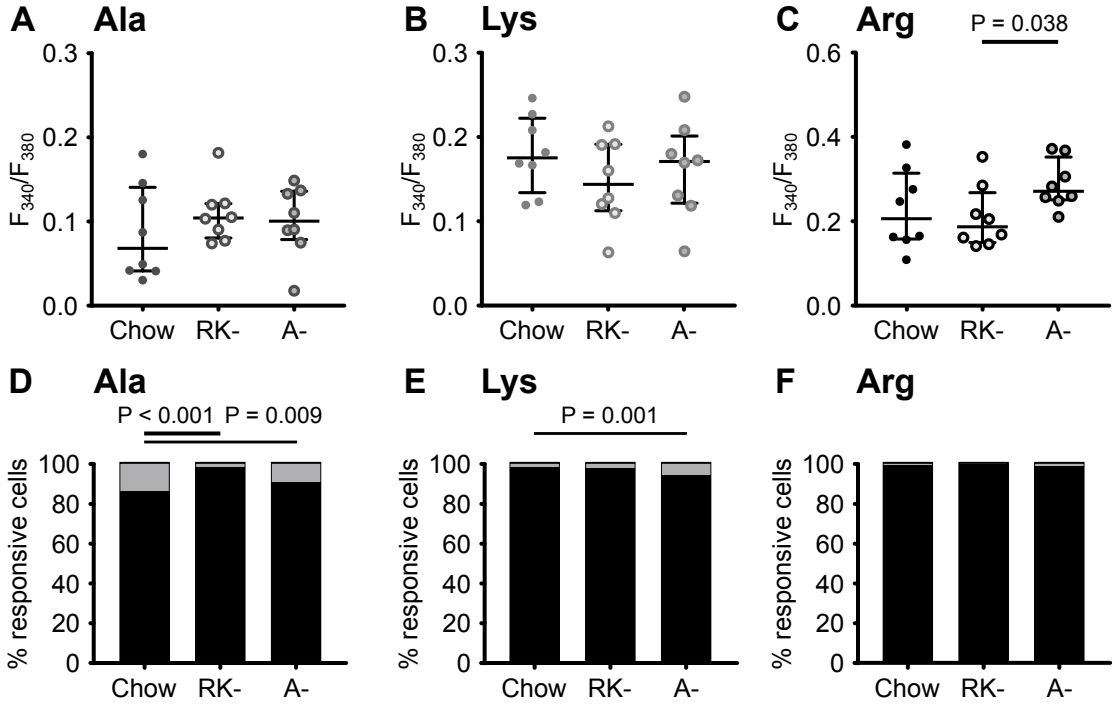

Figure 4.3. Diet deficient in L-arginine and L-lysine increases tanycyte sensitivity to L-alanine. *(**A, D**) 22 hours of the RK- or A- diet increased the proportion of tanycytes responding to L-alanine. (**B, E**) The RK- diet did not affect tanycyte sensitivity to L-lysine, while the A- diet slightly reduced the number of cells responding to L-lysine. (**C, F**) Neither diet affected the responses to L-arginine. There were differences between tanycyte responses to L-arginine on the RK- and A- diets (Student's t test), potentially due to a switch in animal suppliers between the two sets of experiments. The chow dataset is comprised of animals from both suppliers..*

Analysis of the proportion of responsive cells under different diets showed that similarly to fasting, the RK- diet affected tanycyte sensitivity to Ala, increasing the proportion of responsive cells from 85.9% on chow to 97.9% on RK- (Figure 4.3 D). The proportions of cells responding to Arg and Lys under RK- did not change (Figure 4.3 E,F), potentially due to the control responses being so high in each tanycyte that the cells could not produce higher or more reliable responses when challenged with an imbalanced diet; a change in responses to Arg or Lys could still be expected in physiological conditions.

The A- diet also increased the number of tanycytes sensitive to Ala, although to a lesser extent than essential amino acid deficiency – 90.7% of cells responded

on A- (Figure 4.3 D). Unexpectedly, the proportion of cells responding to Lys on the alanine-deficient diet was slightly decreased – from 97.8% on chow to 94.0% on A-.

4.3.4. Essential amino acid-imbalanced diet reduces preference for an L-alanine enriched drink in wild type mice

For an evaluation of behavioural changes potentially occurring due to increased tanycyte sensitivity to Ala, a variation of a 2-bottle preference test was performed. Wild type (C57BL/6J) or *Tas1r1*-null mice were housed in same-sex pairs and given RK- or normal chow for 24 hours in their home cages, then placed in modified cages with 30 g of the same diet, 2 serological pipettes with amino acid solutions (50 mM Ala in the Ala pipette, 25 mM Arg and 25 mM Lys in the Arg+Lys pipette) with blackcurrant flavouring connected to drinking tubes, and a drinking bottle with 50 ml water and blackcurrant flavouring, for the next 24 hours (Figure 4.4 A). Bodyweight, food, Ala, and Arg+Lys intake were measured.

Over the first 24 h in the home cage, female WT mice lost on average 0.106 ± 0.077 g of bodyweight on the RK- diet, which was not different from 0.089 ± 0.051 g lost by the chow fed WT mice. The weight loss persisted throughout the 2-bottle test, but both groups started regaining weight 4 days after the end of the test. Male WT mice had slightly reduced growth in both groups after 24 h in the monitoring cages but gained weight normally as soon as 2 days after the experiment. Overall, there was no marked difference in the weight change between WT mice fed chow and RK- during the 2-bottle test (Figure 4.3 B).

All WT mice ate less RK- than chow (Figure 4.3 C). This may have been due to lower palatability of the diet, in accordance with previous studies which have shown that an essential amino acid imbalanced diet causes avoidance (Leung et al. 1968). Stress related to the novelty of the diet should not have been a factor in this experiment as animals were provided with RK- 24 h prior to the test. Mean food intake did not differ between male and female mice for either diet (data not shown).

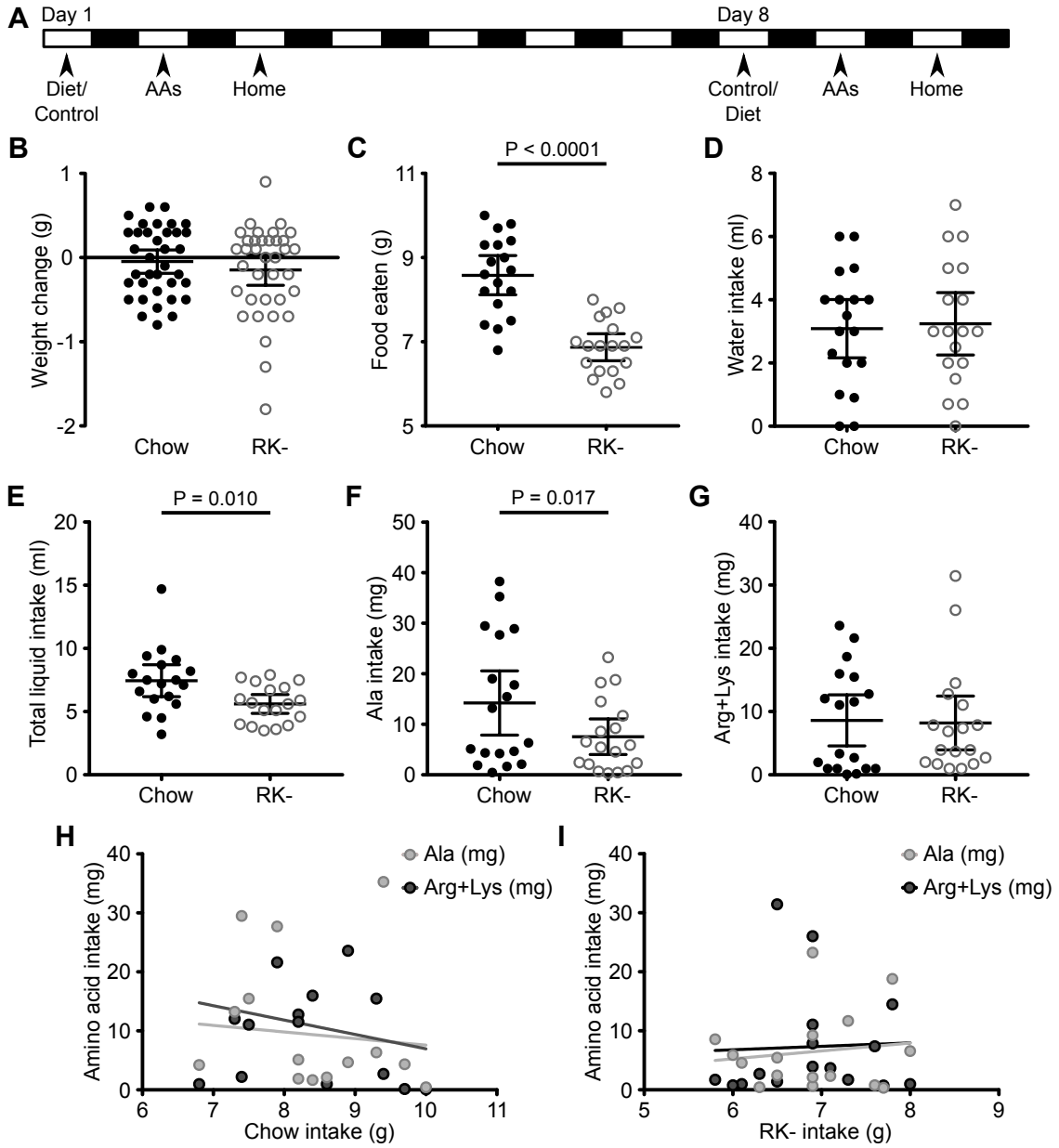

Figure 4.4. Diet deficient in L-arginine and L-lysine reduces preference for an L-alanine-enriched drink. *(**A**) Experimental timeline. (**B**) There were no differences in weight loss between mice fed normal chow and the Arg- and Lys-deficient diet. (**C**) All animals ate less RK- food than normal chow. (**D**) Mice drank a similar amount of water in both dietary conditions. (**E**) Total liquid intake (water, L-alanine-enriched drink, and L-arginine and L-lysine-enriched drink) was reduced on the RK- diet. (**F**) Mouse intake of L-alanine in the form of L-alanine-enriched drink was reduced on the RK- diet. (**G**) The animals showed no changes in preference for the L-arginine and L-lysine-enriched drink on either diet. (**H,I**) Amino acid-enriched water intake in mice fed chow or RK- did not depend on the amount of food consumed. P values given for Student's paired t test.*

Interestingly, while water consumption was nearly identical on both diets, total liquid intake (water, Ala and Arg+Lys solutions combined) was lower under the RK- diet (Figure 4.3 D,E).

As expected from the results of the tanycyte amino acid sensitivity experiment, the RK- diet reduced WT preference for Ala: average Ala intake went from 14.22 ± 3.01 mg per pair of mice on chow to 7.55 ± 1.67 mg per pair on RK- (Figure 4.4 F). The proportion of Ala solution in total liquid consumed was also reduced when WT mice were fed RK- (data not shown). There were no differences in Arg+Lys intake (Figure 4.3 G). As previous results have shown that the RK- diet increases tanycyte sensitivity to Ala, this indicates that the changes in tanycyte Ala detection could potentially influence the choice of amino acid supplements, i.e. more active tanycyte signalling after the intake of the Ala solution would reduce consumption, while unaltered responses to Arg and Lys would produce the same behavioural outcome as in animals on the normal chow diet.

No correlation was observed between the amount of food eaten during the 2-bottle test and amino acid intake (Figure 4.3 H,I). The intake of both amino acid drinks did not vary between males and females on either dietary condition (data not shown).

The design of the experiment did not eliminate the possibility that mice would consume enough Arg and Lys from the water to compensate for the lack of these amino acids in the diet, but the data show that Arg and Lys received from the water was on average 4.1 mg per animal per 24 h, while approximate daily intakes from standard chow (assuming normal consumption of 5 g/day) are 35 mg Arg and 37.5 mg Lys.

4.3.5. *Tas1r1*-null mice show reduced preference for L-alanine independently of essential amino acid intake

When *Tas1r1*-null mice were used for the 2-bottle preference experiment, the results differed from WT. While their food intake was reduced to the same level as WT on RK- (despite supposed inability to taste differences in amino acid content; Figure 4.5 B), the *Tas1r1*-null animals also exhibited significant weight loss (Figure 4.5 C). As both WT and *Tas1r1*-null groups consumed similar amounts of food and liquids (Figure 4.5 D), the *Tas1r1*-null weight loss must be attributed to higher metabolic rate or increased activity.

Unlike WT, *Tas1r1*-null mice did not reduce their Ala intake when put on the RK- diet; however, their mean Ala consumption compared to WT was 7.9 and 3.4 times lower in the Chow and RK- groups, respectively (Figure 4.5 E). Arg and Lys intake remained unaffected (Figure 4.5 F). *Tas1r1*-null animals compensated for the low Ala solution intake by increasing the amount of water they consumed (Figure 4.5 G).

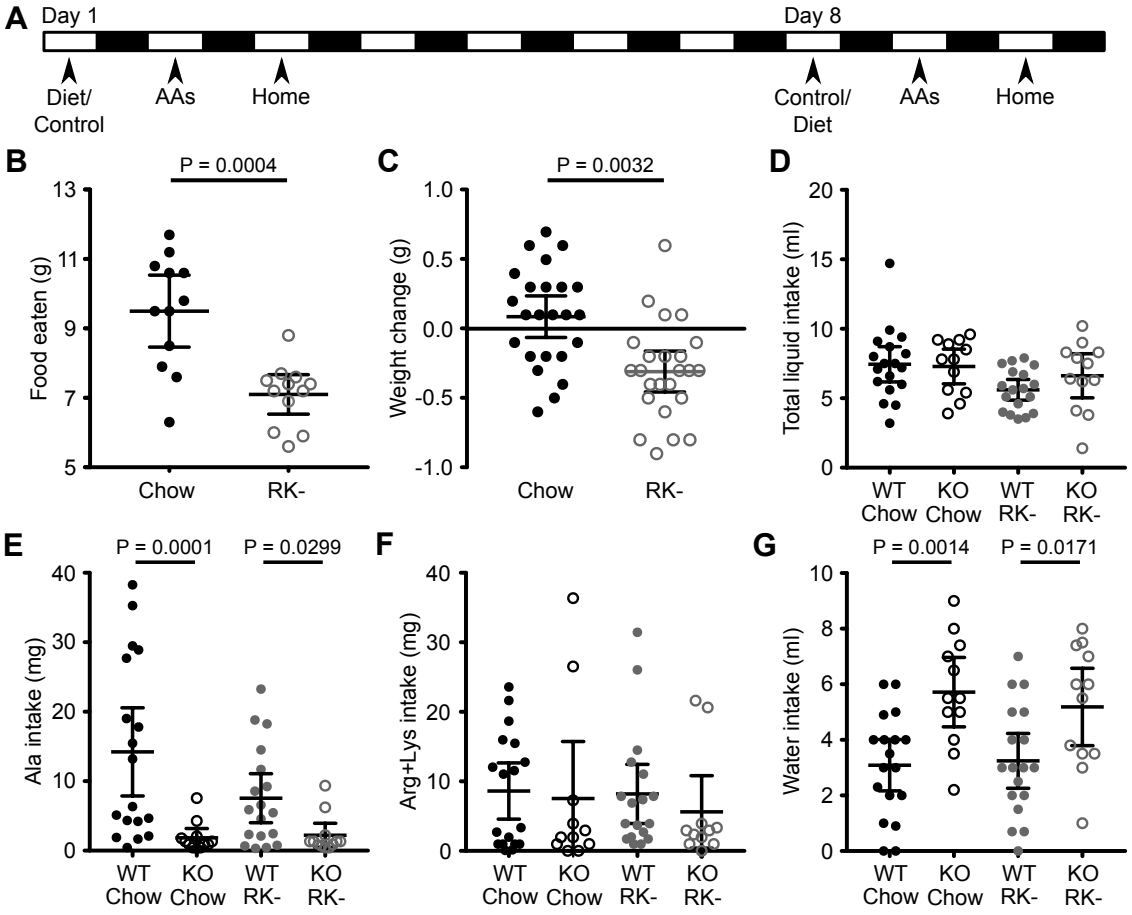

Figure 4.5. Tas1r1-null mice have reduced preference for L-alanine. *(**A**) Experimental timeline. (**B**) When given a diet lacking L-arginine and L-lysine, Tas1r1-null mice consumed less food and (**C**) lost weight. (**D**) Neither loss of the Tas1r1 receptor subunit nor diet affected total liquid intake (consisting of water, alanine solution, and arginine and lysine solution). (**E**) Tas1r1-null mice strongly reduced their alanine intake compared to wild type animals in both chow and essential amino acid-deprived groups. (**F**) Arginine and lysine intake was not affected by diet or genotype. (**G**) Tas1r1-null mice consumed more water to compensate for the reduced intake of the alanine solution. **A, B** Student's paired t test; **E, G** Mann-Whitney U test.*

There were no differences between male and female *Tas1r1*-null mice (data not shown). Moreover, earlier results showed that *Tas1r1*-null animals have unaltered sensitivity to Ala compared to WT (see Figure 3.3) Therefore, tanycyte involvement in the mechanism responsible for *Tas1r1*-null Ala avoidance is unlikely; the behavioural response in potentially mediated by taste

signalling in the tongue or other amino acid detection mechanisms in the brain or gut.

4.3.6. Amino acid-imbalanced diets only have minor effects on mouse metabolism in 24 hours

To further test the effects of dietary amino acid intake on behaviour and energy homeostasis, CLAMS metabolic cages were used. Three types of parameters were chosen for analysis: feeding (intake – amount of food eaten in 3 hours; frequency – number of meals in 3 hours; duration – total time spent at the food hopper in 3 hours), metabolic (all averaged per hour; VCO_2 – CO_2 expenditure, representative of metabolism and activity; Fox – fat oxidation rate; CHOx – carbohydrate oxidation rate; RER – respiratory exchange ratio between CO_2 produced an O_2 used; and heat production), and activity (number of movements per minute around the cage and rearing separately).

NrCAM$^{+/-}$ mice were monitored in CLAMS for 48 hours at a time, with a change of diet occurring in the light cycle after the first 24 hours (Figure 4.6 A). In the first experiment, there was a difference in nutrient composition between the IMB diets and the HP chow (energy from fat/protein/carbohydrate (%) IMB: 9/16/75 vs HP: 18/24/58) which affected mouse feeding and metabolism despite similar caloric content (Figure 4.6). Firstly, mice consumed HP chow less often than A- in the second light phase (the morning after the introduction of the diet; most of the initial light phase was required for habituation, whereas all animals were considered to experience similar stress levels from the onset of the dark phase, hence only the data from the subsequent light phase were used for light phase measurements; Figure 4.6 C). Secondly, the HP diet led to weight loss (Figure 4.6 E) and changes compared to both A- and RK- in most of the metabolic parameters measured in this experiment: reduced CO_2 expenditure (both dark and light phases compared to A-, but only light phase compared to RK-; Figure 4.6 J,N), increased Fox, and reduced RER (Figure 4.6 K,M,O,Q). Light phase activity, including rearing, was increased on chow compared to RK- (Figure 4.6 R,S).

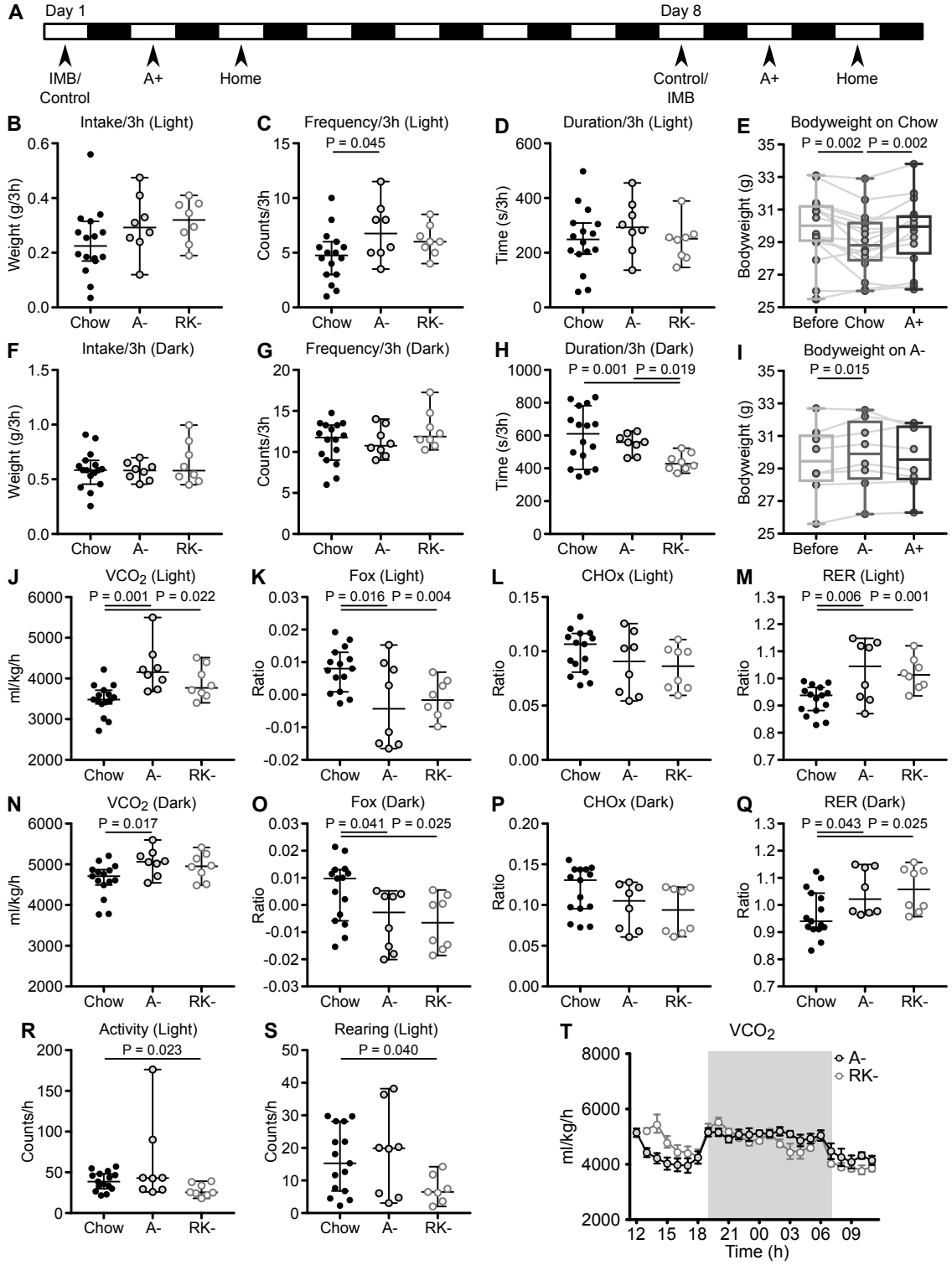

Figure 4.6. Diet composition affects metabolism. *(**A**) Experimental timeline. (**B–D, F–H**) The feeding and (**J–Q**) metabolic effects of different diets on male NrCAM$^{+/-}$ mice. Dark phase feeding duration (**H**) was the only measured parameter that differed between A- and RK- diets. The nutrient composition and fibre contents of the chow led to differences between chow and the two experimental diets in food intake and metabolism. (**E**) Mice lost weight when given chow, but gained weight after the introduction of the Ala enhanced diet. (**I**) Mice gained weight on the A- diet, but returned to previous weight when given food rich in Ala. (**R,S**) Daytime activity was increased in mice receiving chow. Activity during the night was unaffected. (**T**) There was slight but insignificant variation in VCO_2 on A- and RK- in male mice during both light and dark phases. P values given for Student's t test.*

Together, the feeding and metabolic data reveal that high protein chow promotes fat oxidation and increased activity without increasing respiratory rate, while also slightly reducing feeding frequency during the rest period, supposedly due to prolonged satiety provided by the protein and fat in the diet. As the HP chow was composed of natural materials, while the IMB diets were synthetic, the differences in dietary fibre between the diets could also have contributed to the observed changes. Therefore, the experiment was repeated using an appropriate control diet, referred to here as C2. This time, female mice were kept in CLAMS with A-, RK- or C2 for 24 hours before switching to A+.

While the HP chow was unsuitable as a control, comparisons could still be made between the results of the first CLAMS experiments on the amino acid-deficient diets, RK- and A-. One observable difference between male mice deprived of Ala and essential amino acids was a reduction in the duration of feeding in the dark phase on RK- (Figure 4.6 H). Moreover, only the A- diet had a minor effect on bodyweight: male mice gained weight after 24 hours of low Ala diet but returned to their initial weight when given Ala-enhanced food (Figure 4.6 I). No weight changes were observed in female mice in CLAMS.

When analysed hourly, the metabolic data of male mice on A- and RK- showed a non-significant reduction in VCO_2 on RK- in the dark phase, between 0200 and 0500 hours (Figure 4.6 T). There was also an increase in activity on A- at 0100 hours (data not shown). None of these differences were reflected in the phase averages, except for a higher activity level (not including rearing) on A- during the light phase (Figure 4.6 R).

With the redesigned control diet, the only difference between female mice given A- and C2 was feeding duration in the dark: they spent less time at the food hopper when the food was low in Ala (Figure 4.7 G). Meal frequency and the amount of diet consumed were not affected. All feeding and metabolic parameters were similar between RK- and C2, and between RK- and A- (Figure 4.7). These results confirmed the hypothesis that the extensive differences between HP chow and the imbalanced diets were not specific to the amino acids that were lacking in RK- and A-, but mostly occurred due to the composition of the chow diet.

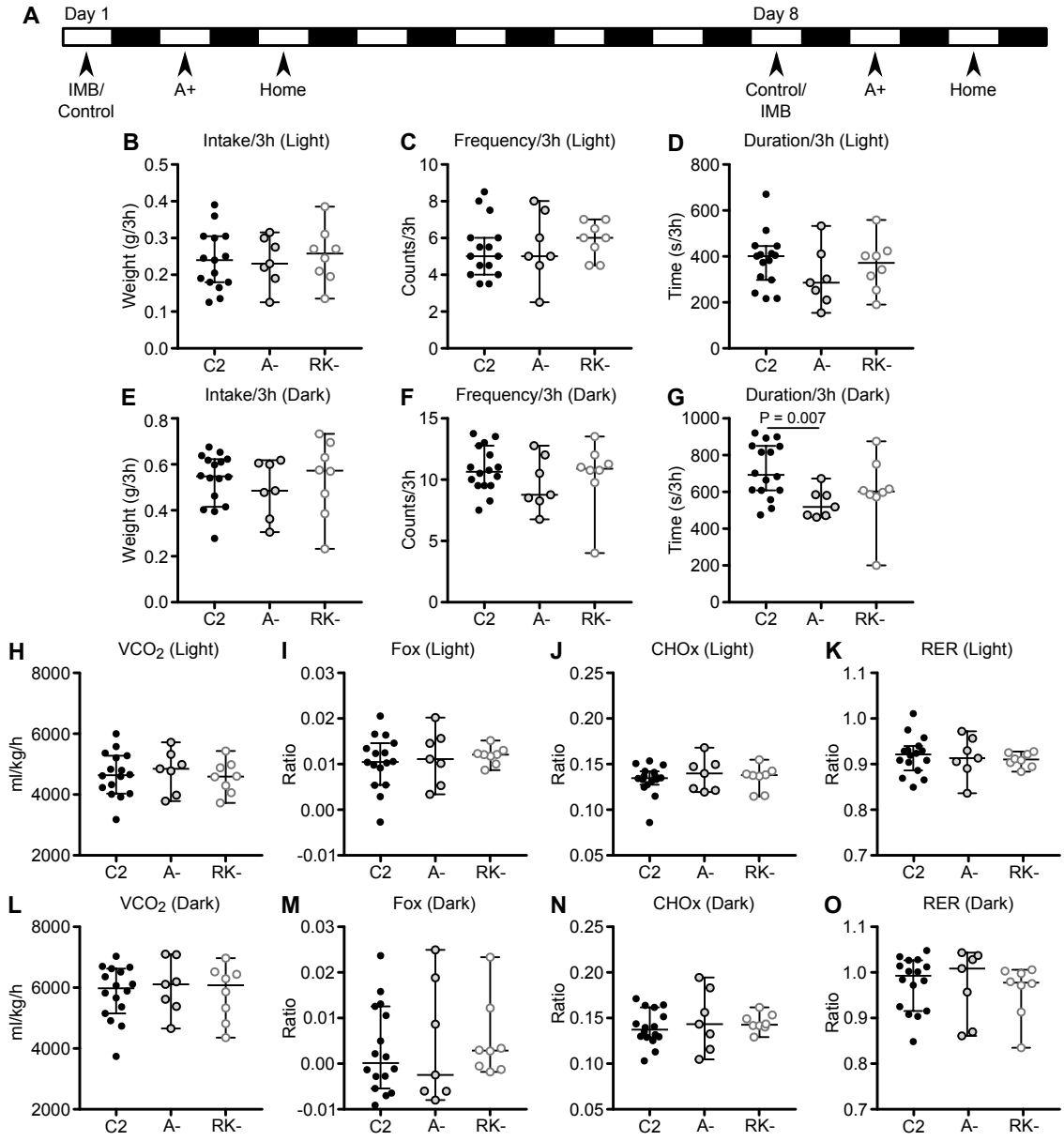

Figure 4.7. Food amino acid content does not affect feeding or metabolism in female mice over 24 hours. *(**A**) Experimental timeline. (**B–G**) Most food intake parameters remained similar on control, alanine-deficient, and arginine- and lysine-deficient diets, except for A-reducing feeding duration in the dark (Student's t test). (**H–O**) Female mouse CO_2 production, fat oxidation, carbohydrate oxidation and respiratory rate were not dependent on diet.*

4.3.7. An alanine-rich diet affects mouse metabolism

After the initial 24 hours, all animals were switched to a high Ala diet to determine the potential effects of variation in Ala intake.

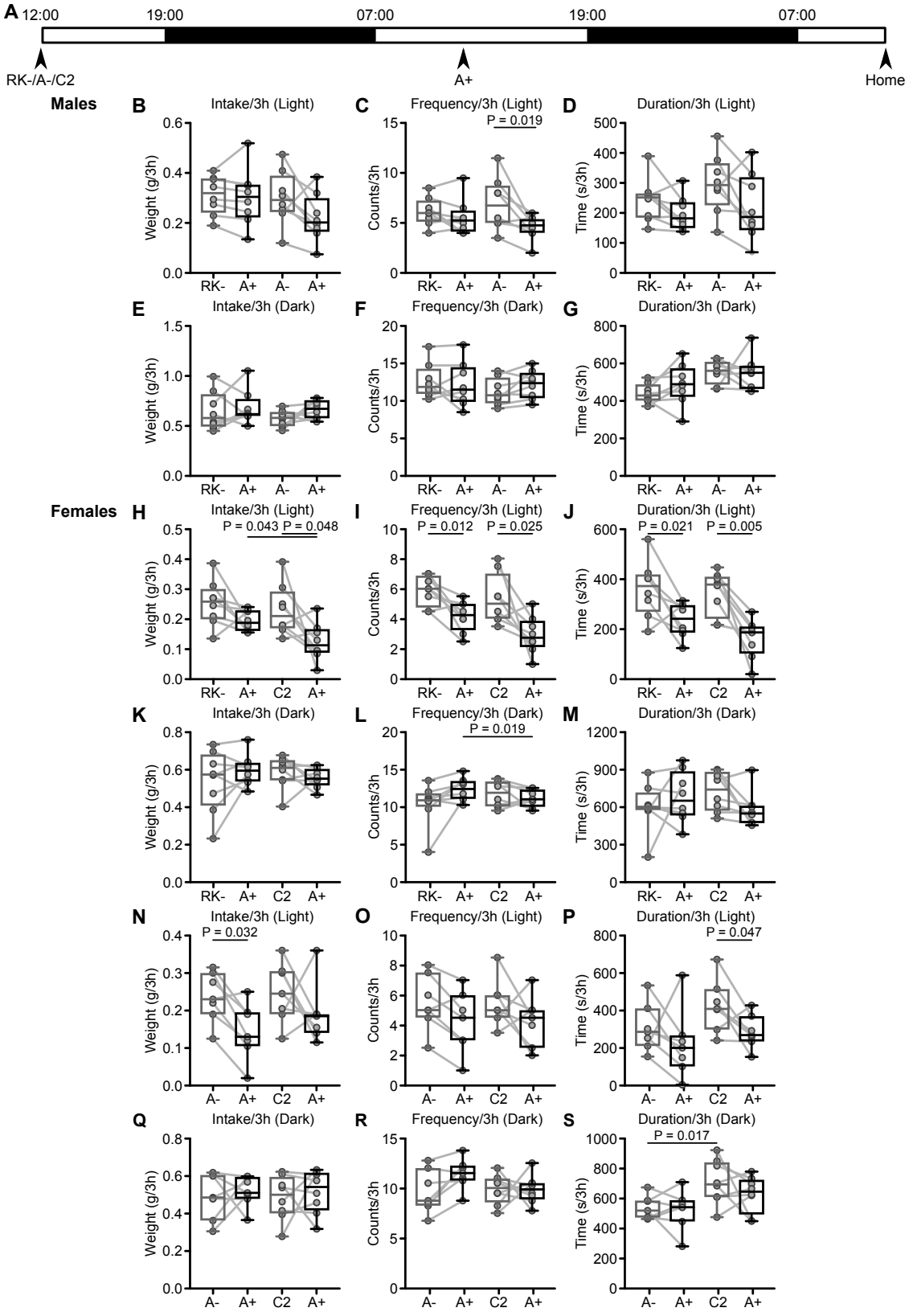

Figure 4.8. Increasing L-alanine content in the diet reduces light phase feeding in male and female mice. *(**A**) Experimental timeline. (**B–G**) Male mice reduced their light phase feeding frequency when switched from alanine-deficient to alanine-enhanced diet, but other feeding-related parameters remained unchanged in both light and dark. (**H –J**) Female mouse exhibited reduced light phase meal frequency and duration when given A+ after both RK- and C2, but only consumed less food after C2. (**K–M**) Female dark phase feeding was unaffected by the diet*

*change. (**N**) Increasing the amount of alanine in food reduced food intake in female mice previously deprived of alanine. (**O, P**) Switching from A- to A+ did not affect meal frequency or duration, but mice switched from C2 to A+ had shorter meals. (**Q–S**) High alanine did not affect dark phase feeding, although mice on C2 exhibited longer meal durations in the initial 24 hours. P values shown for Student's paired t test.*

In the first CLAMS experiment, the switch from the low essential amino acid RK- diet to A+ did not have a high effect on male mouse feeding: there were no differences in food intake at any time of the day (Figure 4.8 B–G, left). Unlike in the 2-bottle experiment, the high Ala content of the A+ diet after exposure to RK- did not reduce food intake. The lack of effect of dietary Ala was potentially due to the diet no longer being imbalanced in Arg and Lys, which perhaps led to increased intake to compensate for the previous lack of essential amino acids. Conversely, switching the diet of Ala-deprived male mice from A- to A+ resulted in reduced feeding frequency on A+ in the light phase (Figure 4.8 C, right), suggesting a higher level of satiety associated with Ala intake.

In females, increasing the amount of Ala in the diet two-fold by switching from C2 to A+ consistently reduced light phase feeding (Figure 4.8 H–J,P, right). The effects of the increase were not always the same as when switched from RK- (Figure 4.8 H,L) or A- (Figure 4.8 N,P,S).

Female mice initially deprived of Arg and Lys were more sensitive than males to A+: light phase feeding frequency and duration were both reduced when switched from RK- to A+; however, these results were also mimicked by mice previously given C2, suggesting that the effect did not specifically require exposure to RK- (Figure 4.8 I,J). Unlike males, when switched from A- to A+, female mice consumed less A+ than A- in the light phase without reducing feeding frequency (Figure 4.8 N).

Essential amino acid deprivation had minor effects on animal activity: male activity was increased by A+ compared to RK- in the light phase but slightly reduced in the dark phase (Figure 4.9 B,E, left), and female dark phase ambulatory activity was decreased under the same conditions (Figure 4.9 F). No changes in activity were observed in animals that initially received A- or C2 (Figure 4.9).

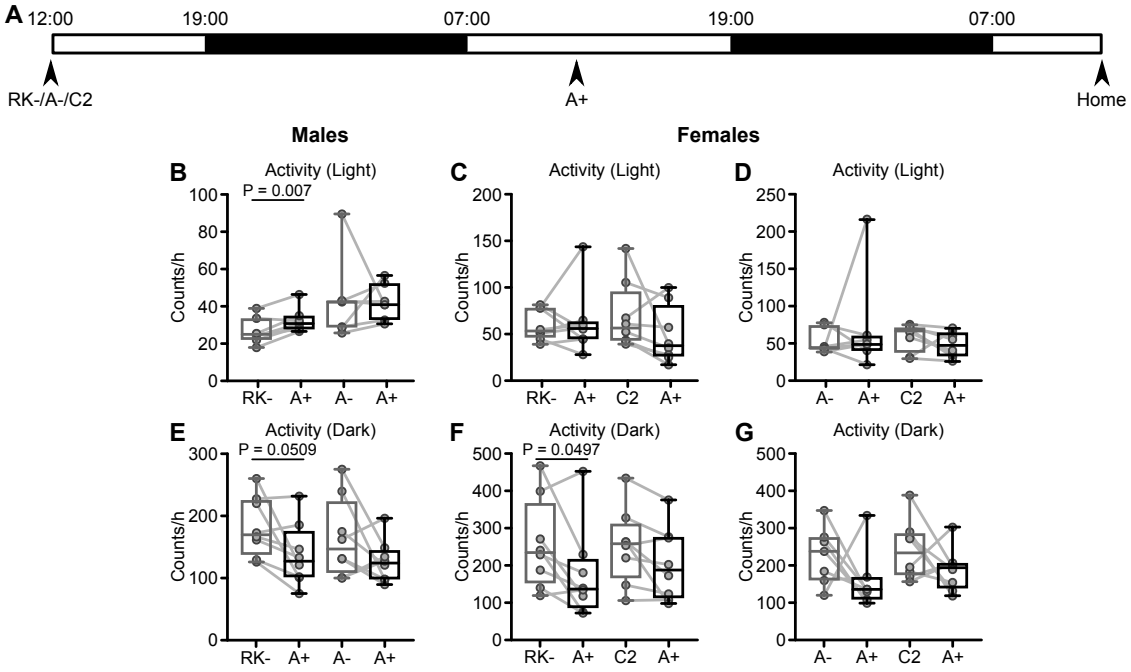

Figure 4.9. Dietary alanine has a minor effect on activity. *(**A**) Experimental timeline. (**B, E**) Male mice deprived of essential amino acids increased light phase activity and reduced dark phase activity when given an alanine-enhanced diet. (**C–D**) Increasing alanine content in the diet did not affect light phase activity in female mice. (**F**) Female mice deprived of arginine and lysine reduced their dark phase activity after receiving a high alanine diet. (**G**) No changes in activity were observed after giving alanine-enriched food to female mice that previously received an alanine-deficient or control diet. P values given for Student's paired t test.*

The only metabolic change in male mice deprived of Arg and Lys after switching from RK- to A+ was an increase in VCO_2 in the dark phase (Figure 4.10 F, left). In females, the switch from RK- to A+ had different effects from males – increased CHOx and heat production in the light (Figure 4.10 L,Q). These results also differed to some extent from the effects seen in animals that switched from C2 to A+: firstly, in the light phase, A+ only decreased VCO_2 in mice previously fed C2 but not RK- (Figure 4.10 J); secondly, light phase Fox was increased and RER was reduced more consistently when switching from C2 rather than RK- to A+ (Figure 4.10 K,M), while light phase heat expenditure was increased only in mice previously fed RK- (Figure 4.10 Q). These differences suggest that essential amino acid deficiency can reduce the effects of high Ala intake. The dependence of the results on previous Arg and Lys intake also eliminates the possibility that the metabolic changes observed on A+ could be due to mouse habituation to the cages.

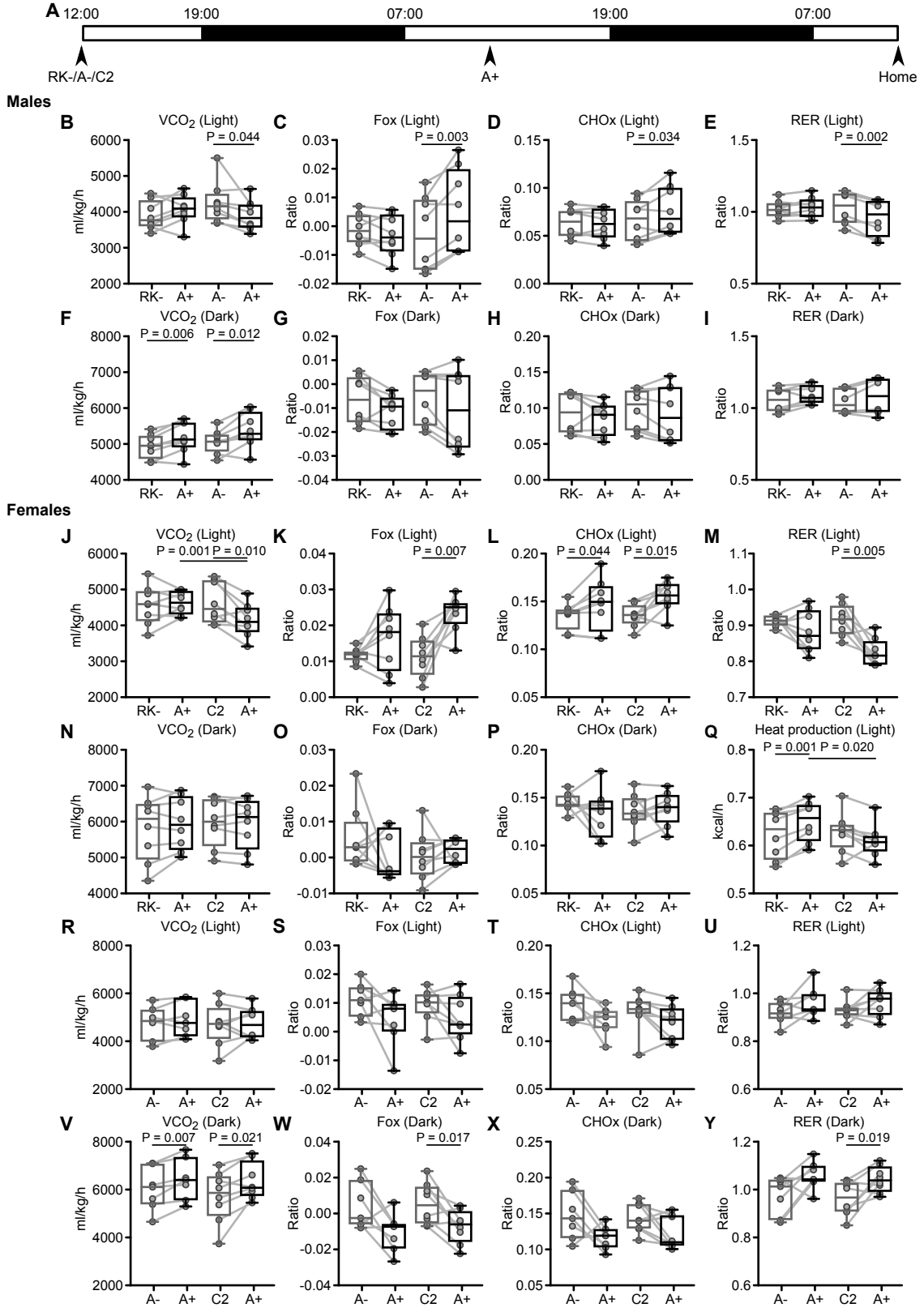

Figure 4.10. An alanine-rich diet affects metabolism. *(**A**) Experimental timeline. (**B–E**) Switching from alanine-deficient to alanine-enriched diet consistently affected male mouse metabolism in the light phase. (**F–I**) An increase in dietary alanine increased male dark phase CO_2 production but did not affect any other dark phase metabolic parameters. (**J–M**) Increasing alanine in the diet had stronger light phase effects on female mice previously fed the control diet than arginine- and lysine-deficient diet, with the exception of (**Q**) heat production, which was increased by A+ only after essential amino acid deprivation. (**N–P**) High alanine diet had no*

*effects on female mice in the dark phase. (**R–U**) Female mice previously deprived of alanine were not affected by high alanine in the light phase. (**V–Y**) In the dark phase, alanine-rich food increased CO_2 production in female mice independently of previous alanine intake, while reducing fat oxidation and increasing respiratory rate only in the control group. P values shown for Student's paired t test.*

Switching the diet of male mice from A- to A+ also resulted in higher VCO_2 in the dark phase (Figure 4.10 F, right), while lower VCO_2 and RER, and higher Fox and CHOx were observed in the light phase (Figure 4.10 B–E, right). Interestingly, the same effects were observed in female mice switching from the C2 diet to A+ in the light phase in the RK- control group (Figure 4.10 J–M, right), but not in female mice switching from A- to A+ or their controls (Figure 4.10 R–U).

On the other hand, after having been deprived of Ala for 24 hours, female mice also exhibited higher VCO_2 in the dark phase when switched to A+, and, in addition, showed a trend for lower Fox (P = 0.051 Student's paired t test; Figure 4.10 V,W, left). The control animals in this group demonstrated the same effects, with the addition of increased RER in the dark (Figure 4.10 V,W,Y, right) The inconsistency in the control results may have been caused by a yet unidentified flaw in the experimental procedure during the A+ experiment; it could also have occurred due to relatively short times that the animals spent on each diet, and adjusting the protocol to increase exposure to each diet could potentially generate a more reproducible outcome. Nevertheless, the similarities of the results of increasing Ala content in males and females suggest a general role for Ala in mouse metabolism.

Taken together, the results of these experiments demonstrate that a diet high in Ala can influence feeding and metabolism by increasing VCO_2 during the night, reducing food intake during the day and increasing fat and carbohydrate oxidation while reducing respiratory rate to compensate for that; however, the effectiveness of increased Ala consumption depends on prior intake of essential amino acids such as Arg and Lys.

4.4. Discussion

Amino acid detection in the hypothalamus has long been associated with decreased food intake (Panksepp and Booth 1971). The experiments described

in this chapter aimed to find potential links between the satiating effect of amino acids with subsequent metabolic adjustments and tanycyte amino acid signalling. At this stage, the results acquired in the feeding and metabolic studies cannot be argued to depend on tanycytes with any certainty, but are rather attempts to determine a phenotype that could later be assigned to changes in tanycyte signalling.

The findings of all the diet-related experiments are summarised in Table 2. Briefly, modifying the diet or removing food from the cages induced changes in tanycyte sensitivity to Ala, food and amino acid supplement intake, bodyweight, activity and metabolism. Interestingly, a diet deficient in Arg and Lys had the same positive effects on the number of tanycytes responding to Ala as fasting; additionally, essential amino acid deficiency lowered food intake in both rats and mice, and reduced mouse consumption of Ala-enriched water. The RK- diet also had an unexpected impact on decreasing the metabolic effects of high Ala intake, otherwise observed in the light phase in mice previously fed a low-Ala or control diets: reduced CO_2 production and respiratory rate and increased fat and carbohydrate oxidation. A low Ala diet, on the other hand, increased feeding time in female mice and induced weight gain in male mice. These results support the notion that amino acid content in the diet can influence the regulation of energy homeostasis in the short term.

4.4.1. Tanycyte sensitivity to L-alanine is diet-dependent

The hypothalamus regulates food intake by adjusting to the constant variations in the metabolic state of the organism. The results obtained in this study demonstrate that hypothalamic tanycytes can alter their signalling to adapt to food availability. An overnight fast or 22 hours on a diet deficient in two essential amino acids, Arg and Lys, was enough for rat tanycytes to increase their sensitivity to Ala, confirming that tanycyte receptor (or downstream target) expression is dependent on metabolic state. Based on these findings, at least a part of diet-induced hypothalamic plasticity could be attributed to tanycyte amino acid signalling.

No.	Fig.	Animals	Sex	Protocol	Diet	Weight	Tanycyte responses	Feeding	Metabolic effects
4.3.1	4.1	SD Rats	M	Ca2+ imaging	Fasting	Loss	More responses to Ala		
4.3.2	4.2	SD Rats	M	Refeeding	Fasting	Loss		Ala does not affect refeeding	
4.3.3	4.3	SD Rats	M	Ca2+ imaging	RK-	Loss	More responses to Ala	Rats eat less RK- than chow	
4.3.4	4.4	C57BL/6J mice	M,F	2-bottle test	RK-	Normal		RK- reduces preference for Ala	
4.3.5	4.5	*Tas1r1* -null mice	M,F	2-bottle test	RK-	Loss		RK- does not affect amino acid preference; *Tas1r1* -null consume less Ala than WT	
4.3.6	4.6	*NrCAM* +/- mice	M	CLAMS	HP	Loss		Changes in all parameters due to low diet compatibility	Changes in all parameters due to low diet compatibility
	4.6	*NrCAM* +/- mice	M	CLAMS	RK-, A-	Gain		RK- reduces feeding time (dark)	A- induces weight gain
	4.7	*NrCAM* +/- mice	F	CLAMS	RK-, A-	Normal		A- increases feeding time (dark)	
4.3.7	4.8 4.9 4.10	*NrCAM* +/- mice	M	CLAMS	RK- to A+	Normal		A+ after RK- reduces feeding duration (light)	A+ after RK- increases VCO2 and reduces activity (dark); increases activity (light)
	4.8 4.9 4.10	*NrCAM* +/- mice	F	CLAMS	RK- to A+	Normal		A+ after RK- reduces feeding frequency and duration (light)	A+ after RK- increases CHOx and heat production (light); reduces activity (dark)
	4.8 4.9 4.10	*NrCAM* +/- mice	M	CLAMS	A- to A+	Gain		A+ after A- reduces feeding frequency (light)	A+ after A- reduces VCO2, RER, increases Fox and CHOx (light); increases VCO2 (dark)
	4.8 4.9 4.10	*NrCAM* +/- mice	F	CLAMS	A- to A+	Normal		A+ after A- reduces food intake (light)	A+ after A- increases VCO2, reduces Fox (dark)

If tanycytes act as a signal of satiety within the hypothalamus to reduce or terminate feeding, changes in tanycyte sensitivity to Ala could serve as an additional mechanism for the avoidance of essential amino acid imbalanced diets in rats (Gietzen et al. 1998). Increased signalling in response to amino acids abundant in food such as Ala would make the animal feel satiated before it has consumed the amount of food that would otherwise be needed to satisfy hunger.

Reflecting this, in the 2-bottle preference test presented in this work, wild type mice consumed less food when given an essential amino acid deficient diet, as well as less water supplemented with Ala, while their preferences for plain water and water supplemented with the limiting amino acids were unaffected. One speculative interpretation of these results could be the occurrence of Ala-specific satiety, reducing Ala intake while allowing the consumption of essential amino acids to remain normal. Interestingly, the Ala satiety seems to be specific to essential amino acid deprivation rather than fasting, as fasted rats did not alter their food intake during refeeding when supplemented with Ala. In *Tas1r1*-null mice, which are less likely to be able to distinguish between different amino acids, the putative Ala-specific satiety is lost, possibly to prevent avoidance of protein-rich foods.

So far, changes in hypothalamic gene expression in response to fasting for two parts of the tanycyte sweet taste signalling pathway have been described: reduced expression of Cx43 (Allard et al. 2014), and increased expression of $P2Y_1$ (Seidel et al. 2006). Low blood glucose levels are also associated with the reorganization of tanycyte tight junctions, leading to greater blood-hypothalamus barrier permeability and allowing blood-borne molecules such as ghrelin easier access to ARC neurons (Langlet et al. 2013). While both Cx43 and $P2Y_1$ also have some involvement in tanycyte amino acid sensing, as demonstrated in Chapter 2, the changes described above seem to be directly related to blood glucose levels, but not overall fasting.

Obesity and high-fat diet, on the other hand, have been shown to reduce the expression of Tas1r3 and G protein subunit α14 in both the hypothalamus and brainstem (Chao et al. 2016). This shows that the taste signalling systems in the

areas of the brain regulating food intake are highly adaptive to changing metabolic states, and the results of the diet-related tanycyte sensitivity experiment include tanycyte amino acid sensing in this adaptation mechanism.

4.4.2. Potential further tanycyte signalling adaptations to dietary changes

In addition to changes in tanycyte sensitivity, under varying feeding conditions tanycyte signals could affect different neuronal populations. Experiments using optogenetic tanycyte stimulation have shown that in *ad libitum* fed mice, the activation of tanycytes expressing CaTCh with flashes of blue light activated nearby NPY and POMC neurons, but following fasting, the effect on POMC neurons was lost (Dr. M. Bolborea, personal communication, September 2017). This effect could be explained by a recent study on POMC neuron sensitivity, which has shown that fasting reduces the excitability of these cells, along with their glucose and leptin sensitivity, due to mitochondrial fission (Santoro et al. 2017). This is also in line with the observation by Kittner and colleagues that P2 receptor stimulation with purine analogues enhanced food intake in food deprived animals (Kittner et al. 2006). If tanycytes were involved in this mechanism, tanycyte purinergic signalling during fasting would only be able to activate NPY neurons, therefore having an orexigenic effect.

Interestingly, in the aforementioned study, *ad libitum* fed animals exhibited increased food intake after specific activation of $P2Y_1$ with adenosine 5′-O-(2-thiodiphosphate), while non-specific ATP analogue 2-methylthioATP had no effect (Kittner et al. 2006). As $P2Y_1$ is associated with tanycyte sweet taste signalling (Benford et al. 2017), the results of $P2Y_1$ activation could be specific to the regulation of glucose intake, while a broader activation of P2 receptors could be involved in the control of total food consumption.

4.4.3. Changes in tanycyte signalling could be linked to metabolism

The two experiments using the CLAMS metabolic cages were performed to further develop the link between tanycyte amino acid sensing and mouse metabolism. Unfortunately, no short-term effects on food intake and metabolism were observed after 24 hour exposure to diets lacking L-alanine or L-arginine and L-lysine. However, switching the food to an L-alanine-enriched diet sufficiently reduced feeding and increased fat and carbohydrate oxidation in

both male and female mice. The effects of the high Ala diet were slightly less in the mice that had previously been starved of essential amino acids; this difference could be related to the previously observed changes in tanycyte Ala sensitivity under the Arg and Lys deficient diet. While it is difficult to confirm that the changes in mouse food intake and energy expenditure were dependent on tanycyte signalling, the ability of tanycytes to detect and distinguish a range of amino acids is a likely candidate for a mechanism that would sense subtle variations in dietary amino acid content.

Mice heterozygous for the *NrCAM* gene were selected for a part of this study with hope that the experiment could be repeated on *NrCAM*-null mice in the future. *NrCAM*-null mice have been shown to have a reduced number of tanycytes, although the exact mechanism of tanycyte loss has not been explained. Early observations of the *NrCAM*-null phenotype have revealed that these mice exhibit reduced body weight from birth, which persists into their adult life (Dr. J. E. Lewis, personal communication, October 2017). If the lean phenotype was a direct result of the absence of tanycytes, this would contrast the hypothesis that tanycyte signalling is predominantly anorexigenic; however, a study using a different line of mice – *Gnasxl*-null (*Gnasxl* codes a variant of the signalling protein $G\alpha_s$, termed $XL\alpha_s$) – with a similarly lean, hypermetabolic phenotype concluded that their mice lost tanycytes and astrocytes due to postnatal undernutrition, which may have led to further compensatory changes in the hypothalamic organisation (Holmes et al. 2016). Perhaps the reason why there is no direct evidence for tanycyte anorexigenic activity in tanycyte-free *NrCAM*-null mice could be similar. A more direct model of tanycyte deletion, preferably one that could be activated postnatally, is required to further explore the role that tanycyte signalling plays in the regulation of metabolism.

A common flaw in research using different diets is an oversimplification of diet composition; for example, studies investigating the effects of HFD may use a well-defined, purified high-fat diet for the experimental group, while giving the control group standard laboratory rodent chow, not taking into account the potential effects of dietary fibre, which can lead to misinterpretations of the collected data (Dalby et al. 2017). The extent of a similar mistake can be seen

in the first CLAMS experiment, where the control chow differed from the synthetic purified IMB diets not only in amino acid content, but also in caloric density, proportions of energy provided by protein, fat and carbohydrate, amount of dietary fibre, smell and, most likely, taste. This oversight was avoided in the second experiment by using a 50:50 mix of ground A+ and A- diets to achieve a control diet with a balanced level of Ala. In the Ca^{2+} imaging experiments, the differences in the nutrient and fibre composition of chow and IMB diets were not relevant as only RK- had an effect on tanycyte sensitivity to Ala, while the responses on the nearly identical A- did not differ from those on chow.

This set of experiments is the first link between tanycyte amino acid sensing and subsequent behavioural adaptations. While the connection between tanycyte Ala sensitivity and mouse dietary choices is at this point highly speculative, the subtle changes observed in this study further reinforce the need for more detailed research on the role of hypothalamic tanycytes in the control of energy homeostasis, not only at the physiological, but also at the behavioural level.

5. Discussion

Tanycyte chemosensitivity has received increasing attention in recent years, and some tanycyte functions involving the detection of hormones and nutrients have been well established. Research into tanycyte nutrient sensing began with the discovery of GLUT2 and glucokinase in tanycytes, which led to an assumption that tanycytes follow the same glucosensing mechanism as pancreatic β cells (García et al. 2003). However, further research revealed that local changes in glucose concentration induce Ca^{2+} and ATP release from tanycytes, which was inconsistent with the glucokinase-dependent mechanism (Frayling et al. 2011, Orellana et al. 2012). The finding that tanycytes also respond to non-metabolisable glucose analogues further strengthened this point of view, while also demonstrating that the sweet taste receptor Tas1r2/Tas1r3 was responsible for the Ca^{2+} and ATP release (Benford et al. 2017).

Glucose represents one of the three basic types of macronutrients, and therefore the question arises whether these cells also possess the ability to detect fatty acids and amino acids. While there is no consensus on the existence of a taste receptor for fatty acids, Tas1r1/Tas1r3 is known as a broadly-tuned amino acid receptor (Nelson et al. 2002). The similarities between the sweet taste receptor and Tas1r1/Tas1r3 prompted the unexpected discovery described in this thesis that tanycytes are sensitive to amino acids. The significance of tanycyte amino acid sensing might exceed that of glucosensing, as amino acids are a central signal of satiety, while glucose has not been demonstrated to have such properties (Panksepp and Booth 1971).

Hypothalamic tanycytes sense L-amino acids in third ventricular CSF using Tas1r1/Tas1r3, mGluR4 and potentially another amino acid receptor yet to be identified. Both of the receptors known so far are GPCRs, and the response pathway indeed follows that of other GPCRs: first, Ca^{2+} is released from the internal stores of the cell; then an ATP signal is sent between the cells via pannexin 1, connexin 43 or CalHM1, as well as down the tanycyte processes and into the hypothalamic nuclei responsible for food intake and energy expenditure. Gustducin, PLC-β2, and IP_3, responsible for the opening of Ca^{2+}

channels for ATP release, have been found in tanycytes by different labs (Orellana et al. 2012, Chao et al. 2016). The same mechanism is known to be used for L-amino acid detection in the tongue.

The data collected during this project revealed several unexpected results. Firstly, there are at least two receptors for L-amino acids. The initial explanation for this was that Tas1r1/Tas1r3 was activated by essential amino acids (such as Arg, Lys), and mGluR4 detected non-essential amino acids (e.g. Ala, Ser), but further experiments with the with the mGluR4 antagonist MAP4 showed some agonist overlap: Lys is an agonist for both Tas1r1/Tas1r3 and mGluR4 in mice. The conclusion is therefore that multiple amino acid detection mechanisms exist in tanycytes due to the physiological importance of amino acids and the signals they elicit: the loss of one mechanism would not cause any significant disruption to the energy homeostasis of the organism as it could be compensated for by the others, as seen in the responses to Arg in male *Tas1r1*-null mice (Fig. 3.3 D). Furthermore, the simultaneous activation of several amino acid receptors could in theory help evaluate the quality of the food that has been ingested: high protein content and a wide range of amino acids represents rich, nutrient dense food, which normally leads to a long-lasting satiating effect.

Secondly, there was sexual dimorphism in mouse responses to some amino acids. Notably, male wild type (C57BL/6J) mice were less sensitive to Arg than females of the same strain. The difference was reversed in *Tas1r1*-null mice, with the responses in females strongly reduced, and males exhibiting an increase. This suggests that male but not female mice have a compensatory mechanism for the loss of the Tas1r1 receptor subunit.

Thirdly, tanycyte responses to Ala, which is not normally considered to be involved in the control of feeding but is known to rapidly increase in concentration in the hypothalamus after feeding (Choi et al. 1999), were dependent on the metabolic state of the animals. Fasting and essential amino acid deprivation increased tanycyte sensitivity to Ala, while the alanine enhanced diet reduced feeding frequency in the light phase in the CLAMS experiments and increased CO_2 expenditure during the dark phase. These findings support the idea that tanycytes alter their signalling to produce an

appropriate response to varying dietary conditions, although definitive evidence linking changes in behaviour to tanycytes is currently missing. The changes in tanycyte signalling were observed after less than 24 hours of access to any diet, or an overnight fast, meaning that the adaptation to dietary challenges is rapid; a long-term study would be required to determine whether chronic intake of different levels of dietary alanine or other amino acids would alter, for example, tanycyte proliferation to further adjust the hypothalamic architecture to the specific conditions.

5.1. Similarities between tanycytes and taste receptor cells

The recent discoveries of a range of taste receptors in hypothalamic tanycytes suggest that tanycytes and taste receptor cells of the tongue might be somewhat alike. The similarities between tanycyte and taste receptor cell amino acid sensing extend beyond the use of the same umami taste receptors. Firstly, in type II taste receptor cells, ATP is used as one of the transmitters to send signals about the taste qualities of a substance to nearby sensory nerve fibres, which use $P2X_2$ and $P2X_3$ receptors to detect it (Finger et al. 2005). ATP also provides positive autocrine feedback onto taste receptor cells by activating P2Y receptors to enhance ATP release (Yasumatsu et al. 2012). The current study has provided sound evidence that tanycytes respond to amino acids with increased intracellular Ca^{2+} and the release of ATP, the spread of which requires several P2X and P2Y receptors. Direct measurements of ATP release from tanycytes using microelectrode biosensors have revealed not only that tanycytes respond to amino acids by producing a wave of ATP, but also that the ATP signal passes into the brain parenchyma. Hypothalamic neurons are known to express both P2X and P2Y receptors that could detect tanycyte signals (Xiang et al. 1998, Wollmann et al. 2005, Seidel et al. 2006, Colldén et al. 2010). Therefore, there is a potential for tanycyte-to-neuron signalling, which would allow tanycytes to inform neurons in the arcuate nucleus and possibly the rest of the hypothalamus about amino acid availability in the third ventricle. Moreover, with responses from different umami taste receptors, the signal could code specific information about which individual amino acids are available, as

the signalling pathways vary depending on which amino acid is applied to tanycytes.

ATP is released from tanycytes via Panx1 and CalHM1 channels, both of which are involved in umami taste signalling in the mouse tongue (Huang et al. 2007, Taruno et al. 2013). There has been contradictory evidence for the two channels in taste receptor cells, as different groups have shown strong effects of disabling either Panx1 or CalHM1 on taste transduction (Dando and Roper 2009, Taruno et al. 2013, Sclafani et al. 2014). The results of this study demonstrate that tanycytes use both Panx1 and CalHM1, and that the two channels seem to be downstream of different umami taste receptors: activation of Tas1r1/Tas1r3 with Arg opens Panx1, while activation of mGluR4 with Ala induces ATP release through CalHM1.

It is not yet fully known how taste receptor cells that are sensitive to different taste modalities but innervated by the same taste nerves keep their signals separate. The contrast between tanycyte sweet taste sensing involving Panx1 and targeting $P2Y_1$ (Benford et al. 2017) and umami taste signalling via Panx1 and CalHM1 targeting multiple P2 receptors is potentially how tanycytes distinguish the information about glucose and amino acid availability. While the difference between sweet and umami taste signalling in tanycytes was unexpected, the possibility of taste-specific signals suggests that tanycytes are more than messengers for food intake and can provide specific information about glucose and various amino acid availability to the hypothalamus. Considering the parallel between tanycytes and taste receptor cells, it is likely that the level of activation of different P2 receptors on taste nerves is also the manner in which taste receptor cells keep their signals separate.

A further shared property of tanycytes and taste receptor cells is that both cell types respond to brain-derived neurotrophic factor (BDNF) and proliferate in adult mice (Givalois et al. 2004, Meng et al. 2015). BDNF is essential for maintaining taste bud innervation and taste cell renewal (Meng et al. 2015).

The detection of circulating nutrients by tanycytes therefore shows remarkable similarity to taste sensing in the taste receptor cells of the tongue, both in the

range of receptors involved and the subsequent downstream signalling including channel-mediated release of ATP. In light of these findings, tanycytes should be regarded as general nutrient sensors in the hypothalamus.

5.2. Tanycytes as amino acid sensors in the brain

Amino acids are a key nutrient, and their constant monitoring in both the brain and periphery is crucial for survival. Moreover, protein is known for its satiating effects (Panksepp and Booth 1971), suggesting that amino acid detection in the areas of the brain regulating food intake and energy expenditure, namely the hypothalamus and the brainstem, is an integral part of the regulation of energy homeostasis.

The discovery that tanycytes can detect amino acids in the third ventricular CSF via at least two receptors is an important advance in understanding the underlying mechanism of energy homeostasis as it reveals that, firstly, tanycytes are likely a part of the hypothalamic anorexigenic system; and secondly, that they might act together with the neural networks in the hypothalamus to regulate food intake and energy expenditure. The communication could occur via the wave of ATP that is released from tanycyte cell bodies and travels down their processes into ARC, as ARC neurons express P2 receptors (Kittner et al. 2006, Xu et al. 2016). The ATP released from tanycytes could activate the anorexigenic POMC neurons directly, or the orexigenic NPY neurons via the inhibitory mTOR pathway as tanycytes have been shown to have a close anatomical association with NPY neurons (Coppola et al. 2007); mTOR activation would reduce NPY secretion and suppress appetite this way.

It is plausible that tanycytes are capable of signalling to both POMC and NPY populations, but the reception of the signals in these neurons is modulated by the metabolic state, giving different meanings to the tanycyte signals depending on the context. For example, in a starved state even a small increase in CSF alanine would allow robust ATP release from tanycytes that would activate NPY neurons (but not POMC as their sensitivity is reduced by fasting; see Santoro et al., 2017) to promote further feeding. The same tanycyte alanine signal in low

essential amino acid conditions would robustly activate POMC neurons, which would in turn prevent overconsumption of a diet that is not beneficial for the animal. This dual signalling would fit the results from two experiments described in this study: in the first one, fasted (therefore the POMC pathway would be blocked, although tanycyte alanine sensitivity is increased) rats given extra alanine did not reduce food intake during refeeding; in the second one, mice deprived of arginine and lysine (POMC active, tanycyte alanine sensitivity increased) consumed less food and avoided alanine.

5.3. A new role for L-alanine in rodent regulation of energy homeostasis

L-alanine is a good indicator of dietary protein intake as it is abundant in most protein rich foods and its concentration increases rapidly after a meal in both blood plasma and the hypothalamus (Choi et al. 1999, Choi et al. 2000, Choi et al. 2001). The results of the food intake and metabolism experiments described in this thesis point towards a potential role for L-alanine detection in the control of energy metabolism.

Although the most direct experiment on the effects of L-alanine on food intake, where rats were supplemented with Ala after a period of fasting and their food consumption was monitored, showed no involvement of Ala in refeeding or weight gain, the other parts of the study provided several instances when the importance of Ala could be established. Firstly, both fasting and essential amino acid starvation increased tanycyte sensitivity to Ala; secondly, mice starved of essential amino acids reduced their Ala intake; and lastly, a diet enhanced in Ala reduced light phase feeding and increased metabolic rate, but mice that had been starved of essential amino acids exhibited these effects to a lesser extent. Increased tanycyte sensitivity to Ala could have mediated the latter two effects, or, otherwise, all the observed effects may have been influenced by the same factor. For example, a global upregulation of mGluR4 would affect both tanycyte responses to Ala and oral Ala intake due to perceived stronger taste. Either way, combined with the fast rate of post-meal changes in blood and brain Ala levels, these results clearly demonstrate that Ala is an important dietary signal in rodents.

The strong Ala-associated satiety signal is not necessarily a positive one; the reduction in Ala intake observed in the above described experiments, as well as *Tas1r1*-null avoidance of Ala-enriched water seen in the second 2-bottle preference test, suggest an aversive effect. Indeed, in both rodents and humans, extensive satiety eventually leads to leptin resistance (Frederich et al. 1995). Moreover, leptin receptor and its downstream signalling pathway share a lot of similarities with detection mechanisms for some inflammatory markers (Anesten et al. 2017). Satiety also reduces motivation and inhibits the reward pathway (Hommel et al. 2006), which is not beneficial for animals. While satiety induced by high Ala sensitivity most likely does not involve increased leptin secretion, it is likely that rodents could associate feeling excessively satiated with the negative effects of leptin, and therefore would learn to avoid Ala.

Although humans and rodents have different dietary needs, feeding patterns and amino acid receptor sensitivity profiles, their neuroendocrine systems responsible for energy homeostasis are regarded as remarkably similar. Interestingly, one human study has shown that L-alanine supplementation reduced subsequent food intake in healthy men (Rogers and Blundell 1994). These findings have unfortunately not received much attention and therefore were never repeated, so the exact role of L-alanine in human energy homeostasis is yet to be revealed.

5.4. Potential role for amino acid sensing in tanycyte neurogenesis

Hypothalamic tanycytes form one of the three currently known neurogenic niches in the adult rodent brain (Lee et al. 2012). Changes in diet have been repeatedly reported to influence hypothalamic neurogenesis (Kokoeva et al. 2005, Li et al. 2014). The newly produced neurons migrate from the wall of 3V into the arcuate nucleus where they are integrated in the POMC/CART and NPY/AgRP circuits (Pierce and Xu 2010).

Neural stem cell proliferation can be induced by P2Y receptor signalling (Lin et al. 2007). The results acquired in the current project and a previous study in our laboratory show that $P2Y_1$ is one of the receptors in tanycytes that is activated by ATP release following glucose and amino acid application. Therefore, it

could be speculated that chronic tanycyte activation with amino acids or glucose over a long time could lead to a higher probability of cell proliferation.

Receiving a high-fat diet for an extended period of time leads to an increase in POMC neuron generation in the male mouse hypothalamus, which may be associated with the G-protein-coupled receptor 40 (Nascimento et al. 2016). In female mice, the effects of high-fat and low-protein diets on neurogenesis are region-specific: tanycyte proliferation is reduced in the arcuate nucleus, while the number of new cells is increased in the median eminence (Lee et al. 2014). It is not yet clear how a low-protein diet could affect tanycyte proliferation in female mice only. However, considering the data presented in this thesis, it could be speculated that tanycyte amino acid sensing via umami taste receptors could be involved in the maintenance of normal levels of neurogenesis in the hypothalamus, and that a reduction in protein content in the diet would alter the number of newly generated neurons in order to adapt to such changes.

Direct tanycyte-to-neuron signalling about amino acid availability together with amino acid and ATP-induced tanycyte proliferation into arcuate neurons could represent a short-term and a long-term role for tanycytes in the control of energy homeostasis.

5.5. Future work

The research described here has provided the first details of tanycyte amino acid signalling to the metabolic circuits of the hypothalamus. Nevertheless, further studies will be needed in order to elaborate the full tanycyte amino acid sensing pathway and the impact of tanycyte signalling on food intake and energy expenditure.

First of all, dietary mixtures of L-amino acids at physiological post-meal concentrations should be tested on tanycytes to determine whether tanycyte signalling occurs in normal conditions, or if the mechanism is only in place for situations where amino acid levels in the third ventricle exceed regular levels, e.g. inflammation or increased hypothalamic permeability due to high ghrelin. The dietary amino acid mixture would be a useful tool to investigate general tanycyte signalling, as some amino acids in the solution would taste sweet or

bitter and would therefore activate a wider range of receptors than the umami-tasting amino acids used so far.

To date, tanycyte responses to amino acids have been tested in chow-fed, fasted and L-arginine and L-lysine-deprived animals. It would be interesting to see how amino acid detection is affected by high-protein and high-fat diets, as well as check for changes in tanycyte signalling in diet-induced and genetic obesity, and a rodent model of anorexia nervosa. Foetal metabolic programming is another currently popular topic, so investigating the involvement of tanycyte amino acid sensing in this complex process would be a worthwhile project.

In vivo Ca^{2+} imaging is a cutting-edge technique that has proved to be valuable in tanycyte signalling research. Initial experiments in our laboratory have shown an increase in tanycyte signalling in freely behaving animals around 20 minutes after ingestion of a high-protein, high-sucrose chow pellet (T. Sotelo Hitschfeld, personal communication, August 2017). More specific tanycyte signals can be obtained by using diets that differ in their amino acid contents, as well as enriching drinking water with amino acids or other nutrients.

To test if the effects of knocking out the gene coding the Tas1r1 subunit of the umami taste receptor Tas1r1/Tas1r3 are tanycyte-specific, a genetic strategy will have to be developed to rescue *Tas1r1* expression exclusively in tanycytes. These mice will then be used to repeat all the experiments that previously included the *Tas1r1*-null animals, and the results will be compared to both full knock-out and wild type mice. If the responses in the newly generated mice are similar to wild type, this will suggest that tanycytes are directly responsible for the differences observed earlier; however, if the mice expressing *Tas1r1* in tanycytes exhibit results closer to the *Tas1r1*-null mice, it will be clear that tanycytes only play a partial role in central amino acid detection.

In terms of the metabolic studies, while the results presented here are showing a hint of change in food intake, activity and metabolic rate, longer exposure to the amino acid modified diets could yield more reliable results. A study on chronic intake of the L-alanine-deficient and L-alanine-enriched diets has been

designed and is currently ongoing. The study involves the mice consuming either L-alanine-deficient or control diet for a week, then switching to the L-alanine-enriched food for another week, as they are monitored in the metabolic cages at the start and end of each dietary trial. This study will hopefully provide more insight into the role of L-alanine in the regulation of mouse metabolism.

The rat refeeding experiment that aimed to determine the effects of L-alanine supplementation on hunger could be repeated using an alanine-enriched diet instead of alanine-supplemented water to ensure even consumption of L-alanine between experimental groups.

An exciting but presently unlikely study would involve a culture of human tanycytes. The possible discovery of human tanycyte sensitivity to nutrients would provide the essential link to make the results obtained from rodents in this project translatable and further increase the importance of tanycyte research in the context of food intake and metabolism.

6. Conclusions

This dissertation provides novel insight into the role of hypothalamic tanycytes in the regulation of energy homeostasis, exploring in detail tanycyte sensitivity to dietary amino acids via different receptors under a range of conditions. More precisely, the results of the experiments described here have shown that tanycytes respond to a range of non-aromatic L-amino acids in a Ca^{2+} and ATP dependent manner, and Tas1r1/Tas1r3 and mGluR4 are two of the receptors involved in the responses. Altering the animal diets has also revealed that tanycyte sensitivity to L-alanine is dependent on the metabolic status of the subject. And finally, behavioural experiments have drawn a link between tanycyte amino acid sensing, metabolism and behaviour.

The findings that emerged from this work form a new piece of knowledge in the complicated subject of hypothalamic control of energy homeostasis. A more detailed understanding of how food intake and energy expenditure are regulated in the brain may lead to the development of new strategies for overcoming the obesity epidemic and other metabolic disorders.

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Appendix

Chapter 2 data

Figure	Treatment	Trial	Med	Q1	Q3	Slices	Animals
2.6 C	Amino acids	5 mM Arg	0.298	0.219	0.415	6	
		5 mM Lys	0.310	0.193	0.448	6	
		5 mM Ser	0.059	0.045	0.194	4	
		10 mM Ser	0.186	0.089	0.219	5	
		5 mM Ala	0.018	0.015	0.048	4	
		10 mM Ala	0.265	0.103	0.489	12	
		5 mM Pro	0.014	0.001	0.089	3	
		10 mM Pro	0.070	0.037	0.099	10	
2.7 B	TTX	Arg	0.379	0.262	0.512	7	2
		Drug + Arg	0.251	0.131	0.414		
		Wash + Arg	0.168	0.144	0.288		
2.8 B	MRS2500	Arg	0.456	0.391	0.491	7	2
		Drug + Arg	0.346	0.280	0.414		
		Wash + Arg	0.286	0.189	0.457		
C	PPADS	Arg	0.222	0.180	0.442	4	3
		Drug + Arg	0.139	0.097	0.412		
		Wash + Arg	0.137	0.125	0.153		
	Suramin	Arg	0.309	0.138	0.388	6	2
		Drug + Arg	0.177	0.112	0.290		
		Wash + Arg	0.241	0.165	0.420		
	BBG	Arg	0.501	0.339	0.613	5	3
		Drug + Arg	0.404	0.245	0.488		
		Wash + Arg	0.249	0.164	0.403		
F	PPADS/MRS	Arg	0.237	0.200	0.324	4	3
		Drug + Arg	0.104	0.064	0.140		
		Wash + Arg	0.180	0.149	0.291		
	Suramin/MRS	Arg	0.320	0.235	0.404	5	3
		Drug + Arg	0.123	0.095	0.168		
		Wash + Arg	0.177	0.142	0.291		
	BBG/MRS	Arg	0.434	0.408	0.526	7	4
		Drug + Arg	0.217	0.202	0.347		
		Wash + Arg	0.258	0.244	0.294		
2.11 A	Gap26	Arg	0.399	0.295	0.427	10	3
		Gap26 + Arg	0.234	0.095	0.421		
	10panx	Arg	0.233	0.220	0.406	11	4
		10panx + Arg	0.175	0.070	0.271		
	Control	Arg	0.289	0.226	0.467	13	7
		After 30 min	0.319	0.224	0.372		
C	RuR	Arg	0.371	0.246	0.536	5	
		Drug + Arg	0.313	0.184	0.385		
		Wash + Arg	0.181	0.123	0.242		
D	RuR Ala ATP	Ala	5.357	4.659	10.830	5	
		Drug + Ala	2.958	2.463	5.543		
		Wash + Ala	3.110	2.818	6.720		
2.11 E	RuR Ala	Ala	0.247	0.203	0.490	5	
		Drug + Ala	0.156	0.045	0.187		
		Wash + Ala	0.225	0.095	0.325		

Chapter 3 data

Figure	Treatment	Trial	Med	Q1	Q3	Slices	Animals
3.1 C	IMP	Arg	0.097	0.048	0.111	14	4
		Drug + Arg	0.110	0.094	0.201		
		Wash + Arg	0.074	0.04	0.158		
E	IMP	Lys	0.044	0.033	0.062	5	3
		Drug + Lys	0.056	0.043	0.099		
		Ala	0.053	0.043	0.114	5	2
		Drug + Ala	0.063	0.049	0.109		
		Ser	0.078	0.029	0.082	5	1
		Drug + Ser	0.038	0.031	0.063		
3.3 A	Tas1r1-null	WT Ala	0.045	0.023	0.054		23
		KO Ala	0.043	0.032	0.073		20
		WT Lys	0.111	0.073	0.153		24
		KO Lys	0.067	0.044	0.116		23
		WT Arg	0.153	0.081	0.205		23
		KO Lys	0.140	0.062	0.224		21
C	Female KO	WT Ala	0.051	0.023	0.082		9
		KO Ala	0.038	0.036	0.054		7
		WT Lys	0.108	0.053	0.131		8
		KO Lys	0.050	0.030	0.067		11
		WT Arg	0.229	0.180	0.307		8
		KO Arg	0.054	0.047	0.127		8
D	Male KO	WT Ala	0.045	0.023	0.054		15
		KO Ala	0.048	0.027	0.078		13
		WT Lys	0.114	0.080	0.179		16
		KO Lys	0.088	0.051	0.123		12
		WT Arg	0.107	0.064	0.172		15
		KO Arg	0.190	0.125	0.253		13
3.4 A	4-CPG	Ala	0.052	0.044	0.082	7	4
		Drug + Ala	0.021	0.011	0.030		
		Wash + Ala	0.019	0.010	0.062		
		Lys	0.122	0.073	0.264	5	3
		Drug + Lys	0.074	0.058	0.142		
		Wash + Lys	0.061	0.050	0.127		
		Arg	0.125	0.089	0.182	5	4
		Drug + Arg	0.062	0.032	0.215		
		Wash + Arg	0.056	0.051	0.127		
D	MAP4	Ala	0.041	0.023	0.067	9	6
		Drug + Ala	0.015	0.01	0.025		
		Wash + Ala	0.025	0.016	0.036		
		Lys	0.157	0.108	0.236	8	7
		Drug + Lys	0.081	0.051	0.124		
		Wash + Lys	0.105	0.079	0.147		
		Arg	0.162	0.118	0.261	6	5
		Drug + Arg	0.091	0.074	0.151		
		Wash + Arg	0.072	0.059	0.111		
E	MAP4 + KO	Ala	0.042	0.029	0.072	6	6
		Drug + Ala	0.017	0.008	0.019		
		Wash + Ala	0.018	0.012	0.032		
		Lys	0.081	0.057	0.126	6	6
		Drug + Lys	0.069	0.041	0.084		
		Wash + Lys	0.060	0.043	0.101		
		Arg	0.179	0.109	0.244	6	6
		Drug + Arg	0.142	0.073	0.178		
		Wash + Arg	0.146	0.052	0.164		

Chapter 4 data

Figure	Treatment	Trial	Mean	SEM	N
4.1 A	Fasting	Chow Ala	0.073	0.007	6
		Fasted Ala	0.102	0.018	8
B	Fasting	Chow Lys	0.160	0.126	6
		Fasted Lys	0.036	0.028	8
C	Fasting	Chow Arg	0.307	0.049	6
		Fasted Arg	0.268	0.063	8
D	Fasting (%)	Chow Ala	67.31		566
		Fasted Ala	90.33		455
E	Fasting (%)	Chow Lys	85.91		369
		Fasted Lys	87.10		465
F	Fasting (%)	Chow Arg	97.35		378
		Fasted Arg	96.40		445
4.2 A	Fasting weight	Fed	4.458	0.554	12
		Fasted	-7.008	0.588	12
B	Refeeding weight	Fed + Pro	5.483	0.526	6
		Fed + Ala	5.400	0.402	6
		Fasted + Pro	10.880	1.214	6
		Fasted + Ala	10.750	1.272	6
C	Food intake 4h	Fed + Pro	1.15	0.35	6
		Fed + Ala	0.98	0.26	6
		Fasted + Pro	4.08	0.32	6
		Fasted + Ala	4.25	0.50	6
D	Food intake 8h	Fed + Pro	1.85	0.39	6
		Fed + Ala	2.10	0.25	6
		Fasted + Pro	5.82	0.23	6
		Fasted + Ala	5.65	0.72	6
E	Food intake 24 h	Fed + Pro	14.20	2.24	6
		Fed + Ala	17.60	2.75	6
		Fasted + Pro	15.40	1.24	6
		Fasted + Ala	15.10	1.70	6
4.3 A	RK-/A- diet	Chow Ala	0.088	0.020	8
		RK- Ala	0.110	0.012	8
		A- Ala	0.101	0.015	8
B	RK-/A- diet	Chow Lys	0.180	0.016	8
		RK- Lys	0.147	0.018	8
		A- Lys	0.161	0.020	8
C	RK-/A- diet	Chow Arg	0.229	0.034	8
		RK- Arg	0.211	0.026	8
		A- Arg	0.289	0.020	8
D	RK-/A- diet (%)	Chow Ala	85.86		601
		RK- Ala	97.91		575
		A- Ala	90.69		612
E	RK-/A- diet (%)	Chow Lys	97.84		556
		RK- Lys	97.66		555
		A- Lys	93.99		632
F	RK-/A- diet (%)	Chow Arg	99.15		585
		RK- Arg	99.64		561
		A- Arg	98.80		585
4.4 B	RK- + AA solutions bodyweight	Chow	-0.047	0.068	36
		RK-	-0.147	0.089	36
C	RK- + AA solutions food intake	Chow	8.58	0.221	18*
		RK-	6.87	0.152	18*
D	RK- + AA solutions water consumption	Chow	3.090	0.438	18*
		RK-	3.240	0.468	18*
E	RK- + AA solutions total liquid intake	Chow	7.444	0.601	18*
		RK-	5.606	0.354	18*
F	RK- + AA solutions	Chow	14.220	3.008	18*

	Ala intake	RK-	7.547	1.669	18*
G	RK- + AA solutions	Chow	8.604	1.916	18*
	Arg+Lys intake	RK-	8.189	2.016	18*
4.5 B	KO RK- + AA solutions	Chow	9.50	0.472	12*
	food intake	RK-	7.10	0.258	12*
C	KO RK- + AA solutions	Chow	0.088	0.072	12*
	bodyweight	RK-	-0.308	0.071	12*
D	KO RK- + AA solutions	WT Chow	7.444	0.601	18*
	total liquid intake	KO Chow	7.283	0.568	12*
		WT RK-	5.606	0.354	18*
		KO RK-	6.617	0.720	12*
E	KO RK- + AA solutions	WT Chow	14.220	3.008	18*
	Ala intake	KO Chow	1.847	0.605	12*
		WT RK-	7.547	1.669	18*
		KO RK-	2.210	0.789	12*
F	KO RK- + AA solutions	WT Chow	8.604	1.916	18*
	Arg+Lys intake	KO Chow	7.539	3.676	11*
		WT RK-	8.189	2.016	18*
		KO RK-	5.609	2.340	11*
G	KO RK- + AA solutions	WT Chow	3.090	0.438	18*
	Water consumption	KO Chow	5.717	0.567	12*
		WT RK-	3.240	0.468	18*
		KO RK-	5.183	0.632	12*

CLAMS data

Figure	Treatment	Parameter	Diet	Mean	SEM	N
4.6 B	A-/RK- males	Food intake L	Chow	0.235	0.031	16
			A-	0.302	0.038	8
			RK-	0.313	0.028	6
C		Meal frequency L	Chow	4.69	0.588	16
			A-	6.94	0.933	8
			RK-	6.13	0.498	8
D		Meal duration L	Chow	247.1	28.82	16
			A-	296.4	34.63	8
			RK-	241.7	26.09	8
E		Bodyweight A-	Before	29.49	0.780	8
			A-	29.88	0.767	8
			A+	29.64	0.704	8
F		Food intake D	Chow	0.586	0.042	16
			A-	0.574	0.029	8
			RK-	0.648	0.068	8
G		Meal frequency D	Chow	11.16	0.639	16
			A-	11.22	0.640	8
			RK-	12.66	0.822	8
H		Meal duration D	Chow	594.2	42.17	16
			A-	552.1	21.48	8
			RK-	437.9	18.34	8
I		Bodyweight Chow	Before	29.86	0.537	16
			Chow	28.98	0.479	16
			A+	29.63	0.529	16
J		VCO2 L	Chow	3479	97.5	15
			A-	4254	205.6	8
			RK-	3897	141.4	8
K		Fox L	Chow	0.008	0.002	15
			A-	-0.003	0.005	8
			RK-	-0.001	0.002	8
L		CHOx L	Chow	0.100	0.005	15
			A-	0.088	0.010	8
			RK-	0.085	0.007	8
M		RER L	Chow	0.921	0.014	15
			A-	1.027	0.040	8
			RK-	1.017	0.021	8
N		VCO2 D	Chow	4613	110.0	15
			A-	5061	115.6	8
			RK-	4940	117.8	8
O		Fox D	Chow	0.005	0.003	15
			A-	-0.006	0.004	8
			RK-	-0.007	0.004	8
P		CHOx D	Chow	0.117	0.008	15
			A-	0.098	0.010	8
			RK-	0.093	0.010	8
Q		RER D	Chow	0.968	0.022	15
			A-	1.048	0.030	8
			RK-	1.057	0.029	8
R		Activity L	Chow	39.31	2.944	15
			A-	59.91	18.110	8
			RK-	28.26	2.731	8
S		Rearing L	Chow	16.15	2.578	15
			A-	18.56	4.810	8
			RK-	7.41	1.629	7
4.7 B	A-/RK- females	Food intake L	C2	0.238	0.021	15
			A-	0.236	0.025	7
			RK-	0.253	0.027	8

	C		Meal frequency L	C2	5.40	0.406	15
				A-	5.50	0.707	7
				RK-	5.88	0.350	8
	D		Meal duration L	C2	382.5	30.69	15
				A-	306.6	48.20	7
				RK-	360.8	39.99	8
	E		Food intake D	C2	0.534	0.028	16
				A-	0.493	0.047	7
				RK-	0.537	0.059	8
	F		Meal frequency D	C2	10.80	0.455	16
				A-	9.64	0.822	7
				RK-	10.38	0.990	8
	G		Meal duration D	C2	715.9	36.80	16
				A-	537.3	29.56	7
				RK-	600.2	67.97	8
	H		VCO2 L	C2	4660	179.0	16
				A-	4770	260.5	7
				RK-	4566	191.8	8
	I		Fox L	C2	0.010	0.001	16
				A-	0.011	0.002	7
				RK-	0.012	0.001	8
	J		CHOx L	C2	0.133	0.004	16
				A-	0.138	0.007	7
				RK-	0.135	0.005	8
	K		RER L	C2	0.922	0.010	16
				A-	0.918	0.017	7
				RK-	0.912	0.006	8
	L		VCO2 D	C2	5829	218.9	16
				A-	6015	336.2	7
				RK-	5818	321.6	8
	M		Fox D	C2	0.003	0.002	16
				A-	0.004	0.005	7
				RK-	0.005	0.003	8
	N		CHOx D	C2	0.139	0.005	16
				A-	0.147	0.013	7
				RK-	0.145	0.003	8
	O		RER D	C2	0.979	0.015	16
				A-	0.976	0.030	7
				RK-	0.964	0.021	8
4.8	B	Switch to A+ food intake	M food intake L	RK-	0.313	0.028	8
				A+	0.304	0.041	8
				A-	0.302	0.038	8
				A+	0.221	0.034	8
	C		M frequency L	RK-	6.13	0.498	8
				A+	5.56	0.637	8
				A-	6.94	0.933	8
				A+	4.50	0.433	8
	D		M duration L	RK-	241.7	26.09	8
				A+	197.4	20.15	8
				A-	296.4	34.63	8
				A+	219.7	39.16	8
	E		M food intake D	RK-	0.648	0.068	8
				A+	0.683	0.061	8
				A-	0.574	0.029	8
				A+	0.665	0.033	8
	F		M frequency D	RK-	12.66	0.822	8
				A+	12.19	1.049	8
				A-	11.22	0.640	8
				A+	12.16	0.671	8

	G		M duration D	RK-	437.9	18.34	8
				A+	486.9	38.95	8
				A-	552.1	21.48	8
				A+	546.7	32.83	8
	H		F RK- intake L	RK-	0.253	0.027	8
				A+	0.194	0.012	8
				C2	0.231	0.030	8
				A+	0.124	0.022	8
	I		F RK- frequency L	RK-	5.88	0.350	8
				A+	4.19	0.365	8
				C2	5.31	0.590	8
				A+	2.94	0.438	8
	J		F RK- duration L	RK-	360.8	39.99	8
				A+	235.4	24.18	8
				C2	344.9	31.07	8
				A+	163.1	27.60	8
	K		F RK- intake D	RK-	0.537	0.059	8
				A+	0.595	0.030	8
				C2	0.583	0.031	8
				A+	0.553	0.019	8
	L		F RK- frequency D	RK-	10.38	0.990	8
				A+	12.34	0.537	8
				C2	11.72	0.605	8
				A+	11.09	0.398	8
	M		F RK- duration D	RK-	600.2	67.97	8
				A+	679.9	72.20	8
				C2	724.7	55.61	8
				A+	575.6	50.22	8
	N		F A- intake L	A-	0.493	0.047	7
				A+	0.516	0.031	7
				C2	0.484	0.042	8
				A+	0.515	0.041	8
	O		F A- frequency L	A-	5.50	0.707	7
				A+	4.21	0.770	7
				C2	5.50	0.598	8
				A+	4.29	0.635	8
	P		F A- duration L	A-	306.6	48.20	7
				A+	220.1	69.46	7
				C2	425.4	53.33	8
				A+	285.4	33.88	8
	Q		F A- intake D	A-	0.236	0.025	7
				A+	0.144	0.028	7
				C2	0.247	0.030	8
				A+	0.191	0.030	8
	R		F A- frequency D	A-	9.64	0.822	7
				A+	11.43	0.582	7
				C2	9.88	0.526	8
				A+	9.88	0.502	8
	S		F A- duration D	A-	537.3	29.56	7
				A+	522.8	49.97	7
				C2	707.1	51.87	8
				A+	626.6	42.82	8
4.9	B	Switch to A+ activity	M activity L	RK-	26.9	2.71	7
				A+	32.7	2.56	7
				A-	43.3	8.31	7
				A+	42.7	3.60	7
	C		F RK- activity L	RK-	60.1	5.95	8
				A+	62.5	12.38	8
				C2	69.4	12.88	8
				A+	49.0	10.88	8

	D		F A- activity L	A-	52.3	6.27	8
				A+	70.2	24.91	8
				C2	58.8	6.50	8
				A+	48.3	5.98	8
	E		M activity D	RK-	184	17.14	8
				A+	137.9	18.05	8
				A-	165.3	22.68	8
				A+	127.4	12.33	8
	F		F RK- activity D	RK-	257.9	43.12	7
				A+	176	43.83	7
				C2	253.2	36.11	8
				A+	204.9	34.87	8
	G		F A- activity D	A-	228.1	29.45	7
				A+	159.7	30.52	7
				C2	242.5	28.07	8
				A+	189.2	20.33	8
4.1	B	Switch to A+ metabolism	M VCO2 L	RK-	3897	141.4	8
				A+	4067	146.2	8
				A-	4254	205.6	8
				A+	3902	149	8
	C		M Fox L	RK-	-0.001	0.002	8
				A+	-0.003	0.003	8
				A-	-0.003	0.005	8
				A+	0.005	0.005	8
	D		M CHOx L	RK-	0.085	0.007	8
				A+	0.083	0.007	8
				A-	0.088	0.010	8
				A+	0.101	0.012	8
	E		M RER L	RK-	1.017	0.021	8
				A+	1.031	0.025	8
				A-	1.027	0.040	8
				A+	0.960	0.045	8
	F		M VCO2 D	RK-	4940	117.8	8
				A+	5185	152.2	8
				A-	5061	115.6	8
				A+	5381	173.4	8
	G		M Fox D	RK-	-0.007	0.004	8
				A+	-0.011	0.002	8
				A-	-0.006	0.004	8
				A+	-0.011	0.006	8
	H		M CHOx D	RK-	0.093	0.010	8
				A+	0.854	0.008	8
				A-	0.098	0.010	8
				A+	0.092	0.014	8
	I		M RER D	RK-	1.057	0.029	8
				A+	1.092	0.022	8
				A-	1.048	0.030	8
				A+	1.083	0.044	8
	J		F RK- VCO2 L	RK-	4566	191.8	8
				A+	4627	112.4	8
				C2	4627	201.8	8
				A+	4143	164.1	8
	K		F RK- Fox L	RK-	0.012	0.001	8
				A+	0.017	0.003	8
				C2	0.011	0.002	8
				A+	0.023	0.002	8
	L		F RK- CHOx L	RK-	0.135	0.005	8
				A+	0.147	0.009	8
				C2	0.135	0.004	8
				A+	0.155	0.006	8

M		F RK- RER L	RK-	0.913	0.006	8
			A+	0.882	0.021	8
			C2	0.915	0.015	8
			A+	0.824	0.013	8
N		F RK- VCO2 D	RK-	5818	321.6	8
			A+	5943	258.1	8
			C2	5965	237.2	8
			A+	5939	250.00	8
O		F RK- Fox D	RK-	0.005	0.003	8
			A+	0.000	0.002	8
			C2	0.000	0.002	8
			A+	0.002	0.001	8
P		F RK- CHOx D	RK-	0.145	0.003	8
			A+	0.134	0.009	8
			C2	0.135	0.007	8
			A+	0.139	0.006	8
Q		F RK- Heat L	RK-	0.624	0.018	8
			A+	0.650	0.015	8
			C2	0.628	0.015	8
			A+	0.609	0.012	8
R		F A- VCO2 L	A-	4770	260.5	7
			A+	4897	271.1	7
			C2	4693	310.2	8
			A+	4750	234.4	8
S		F A- Fox L	A-	0.011	0.002	7
			A+	0.004	0.003	7
			C2	0.009	0.002	8
			A+	0.005	0.003	8
T		F A- CHOx L	A-	0.138	0.007	7
			A+	0.122	0.006	7
			C2	0.131	0.007	8
			A+	0.119	0.006	8
U		F A- RER L	A-	0.918	0.017	7
			A+	0.963	0.025	7
			C2	0.930	0.015	8
			A+	0.963	0.020	8
V		F A- VCO2 D	A-	6015	336.2	7
			A+	6418	327.1	7
			C2	5693	379.2	8
			A+	6338	272.2	8
W		F A- Fox D	A-	0.004	0.005	7
			A+	-0.010	0.004	7
			C2	0.006	0.004	8
			A+	-0.007	0.003	8
X		F A- CHOx D	A-	0.147	0.013	7
			A+	0.116	0.006	7
			C2	0.143	0.007	8
			A+	0.123	0.008	8
Y		F A- RER D	A-	0.976	0.030	7
			A+	1.058	0.022	7
			C2	0.961	0.025	8
			A+	1.042	0.019	8